Characterization of impaired CD8+ T cell responses to Chlamydia trachomatis

Characterization of impaired $CD8^+$ T cell responses to *Chlamydia trachomatis*

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Abstract

Chlamydia trachomatis infection is the most common bacterial sexually transmitted disease in the United States. Irregular screening to identify infected individuals and a lack of sterilizing immunity to *C. trachomatis* has led to a dramatic increase in the number of reported *C. trachomatis* infections over the last twenty years. Repeated infections with *C. trachomatis* lead to serious sequelae such as pelvic inflammatory disease and ectopic pregnancy, which can result in infertility.

It is unclear why the adaptive immune system, specifically the CD8$^+$ T cell response, is unable to protect against subsequent *C. trachomatis* infections. In this dissertation I first describe the endogenous CD8$^+$ T cell response in the genital mucosa during *C. trachomatis* infection. I found that primary *C. trachomatis* infection elicits a robust CD8$^+$ T cell response. However, rechallenge with *C. trachomatis* produces a secondary CD8$^+$ T cell response that is numerically weaker compared to the primary response. I found that depletion of CD8$^+$ T cells prior to primary or secondary infection has no impact on the host's ability to clear *C. trachomatis.* All together these data indicate that CD8$^+$ T cells do not contribute to protecting the host against *C. trachomatis* infection of the genital tract.

In the third chapter I examined the expression of different immuno-inhibitory molecules in the genital tract of *C. trachomatis.* I focused on further

characterizing the expression of the immuno-inhibitory ligand PD-L1. I found that upon infection PD-L1 is highly expressed on epithelial cells of the genital tract and dendritic cells within the draining lymph nodes. Furthermore I show that the receptors for PD-L1, PD-1 and B7-1, are highly expressed on $CD8^+$ T cells after infection has resolved. In the final part of this dissertation I demonstrate that the PD-1/PD-L1 pathway contributes to the defective $CD8^+$ T cell response during *C. trachomatis* infection. Deletion or inhibition of PD-L1 or PD-1 restores the $CD8^+$ T cell response and enhances *C. trachomatis* clearance.

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Dedication

To all the educators who work to improve science literacy.

Chapter One: Introduction

Chlamydia trachomatis, Disease and Epidemiology

Chlamydia trachomatis is the most highly reported cause of sexually transmitted bacterial infections in the United States (1). In the last 20 years, the prevalence of *C. trachomatis* infection has more than doubled in the United States. There are 16 defined serovars of *C. trachomatis.* Infection of the ocular tissue by serovars A-C is the leading cause of preventable blindness worldwide (2). Serovars D-K infect the urogenital tissue and are transmitted sexually. Additionally, serovars L1, L2, L2a and L3 not only infect the urogenital tissue and are spread by sexual contact, but also cause an invasive disease of the lymph nodes (3). Women who contract the sexually transmitted *C. trachomatis* can develop fallopian tube scarring, pelvic inflammatory disease, and ectopic pregnancy, all of which can have severe consequences for fertility (4). Although *C. trachomatis* infections can be cleared with antibiotics, primary *C. trachomatis* infections remain subclinical or "silent" in about 70-80% of infected women and thus often go untreated (4, 5). In some untreated women (20-40%) infection ascends to the fallopian tubes where it can establish a persistent infection and cause pelvic inflammatory disease (6). Persistent infections result in immune mediated tissue scarring and fibrosis, which lead to infertility (6). In addition, even with antibiotic treatment there is evidence for recurrent or persistent infections; recurrent infections can lead to worse outcomes in terms of inflammation and damage of the female reproductive tract (7, 8). The evidence of recurrent infections indicates that the immune system fails to develop an effective memory response that can prevent subsequent infections.

The main challenge in controlling *C. trachomatis* disease is identifying infected individuals and treating them before permanent damage to the reproductive tract occurs.

Current efforts to implement widespread screening to detect infected individuals will likely reduce disease spread. However, screening will not prevent the damage that occurs with multiple infections. A vaccination program in girls before they are sexually active will likely be the most impactful approach in reducing *C. trachomatis* infections and limiting disease sequelae. Because natural immunity to *C. trachomatis* is ineffective, it is necessary to develop a vaccine that could induce protection superior to that acquired from natural infection. In order to develop a successful vaccine, it is essential to understand the life cycle of *C. trachomatis* within the host and how natural immunity fails to prevent recurrent *C. trachomatis* infections. By understanding the failure of natural immunity to *C. trachomatis* we can hopefully develop ways to generate a productive immune response through vaccination. The work of this dissertation is designed to better understand the failure of the adaptive immune system to respond to genital tract infection with *C. trachomatis,* specifically the $CD8^+$ T cell response and the specific mechanisms that play a role in inhibiting a productive $CD8^+$ T cell response.

C. trachomatis **Biology**

C. trachomatis is an obligate intracellular bacterium that depends on the host cell for survival and propagation. *C. trachomatis* transitions between two developmental forms during its biphasic life cycle. The elementary body (EB) form of *C. trachomatis* is able to survive outside of the host cell but exhibits very little metabolic activity. Importantly, EBs are the infectious form of *C. trachomatis*. Upon attaching to an epithelial cell, the EB induces its uptake into the host cell (9). Within a membrane bound

compartment termed the inclusion, the EB differentiates into the other developmental form: the metabolically active, but noninfectious, reticulate body (RB) (**Figure 1-1**).

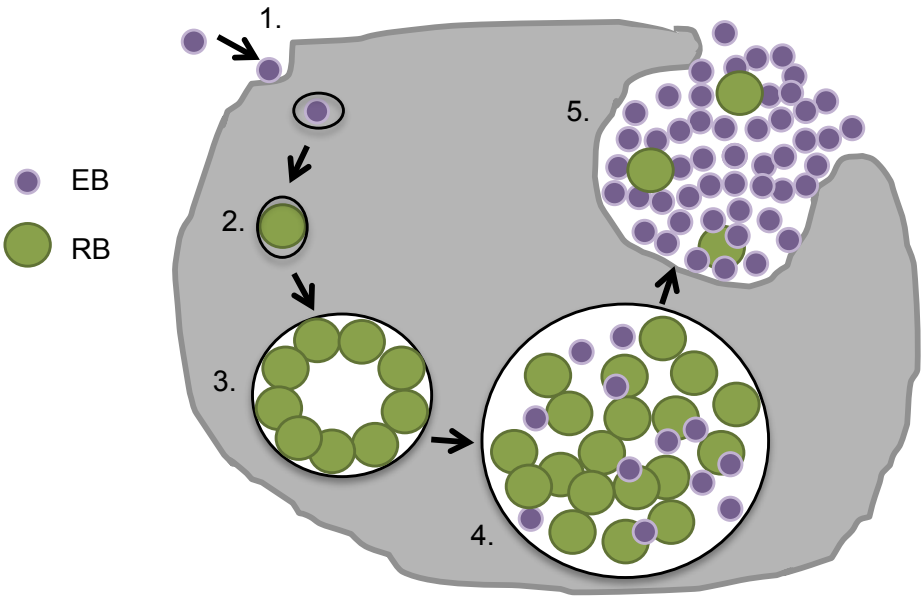

Figure 1-1 Developmental cycle of *C. trachomatis.* *C. trachomatis* has a biphasic life cycle. 1. The metabolically inactive EB (purple) attaches to the host epithelial cell and induces its uptake. 2. The EB transitions into the metabolically active RB within the inclusion. 3. The RBs divide by binary fission within the inclusion. 4. At about 24-26 hours after invasion, RBs begin to differentiate back into elementary bodies. 5. 48-hours post-invasion, EBs are released from the cell and the infectious cycle is repeated.

The membrane of the inclusion is made up of host lipids, mostly derived from the Golgi apparatus and endoplasmic reticulum. In order to replicate, the RBs must scavenge nutrients from the host cell. At the same time, *C. trachomatis* must prevent host cell processes that are meant to degrade foreign and pathogenic entities. Although the RB is secluded within the inclusion, it interacts extensively with the host cell. *C. trachomatis* uses a type III secretion system to inject *C. trachomatis* effectors directly into the host

cytosol. There are about 125 known and predicted *C. trachomatis* type III secreted effectors and the majority of these effectors have no known function (10). However, studies using small-molecule inhibitors of the *C. trachomatis* type III secretion system have indicated that this system is necessary for a productive infection (11, 12). Within the inclusion, the RBs divide extensively by binary fission. Between 24 and 48 hours post infection, through a yet-to-be defined trigger, the RBs differentiate back into EBs. EBs exit the host cell by lysis or extrusion, and can then infect neighboring cells.

Innate Immune Reponses to *C. trachomatis*

Because of *Chlamydia's* unique biphasic lifecycle there are multiple ways in which the immune system can respond to and eliminate *C. trachomatis* from the host tissues. The female genital tract can be subdivided into the lower genital tract including the vagina and cervix, and the upper genital tract including the uterus and fallopian tubes. Upon entering the host through the lower genital tract, *C. trachomatis* is immediately subjected to innate immune responses, which are the primary defenses against infection. Commensal bacteria that colonize the vaginal tissue provide a first line of defense against pathogens. The commensal *Lactobacilli* species produce lactic acid, which keeps the pH of the vaginal tissue between 3.5 and 5 (13). Studies on the effectiveness of topical microbicides have indicated that exposure of *C. trachomatis* EBs to a solution with a pH of 5.0 leads to reduced infectivity, indicating that *C. trachomatis* might be susceptible to the low pH of the vaginal tissue (14). However, the fact that *C. trachomatis* is generally transmitted by coitus makes it likely that *C. trachomatis* can overcome this inhibition.

The cells that line the cervix produce thick mucus that provides a barrier to both sperm and pathogens. Studies have shown that the mucus produced by the cervical epithelial cells also contains the antimicrobial molecules lysozyme and secretory leukocyte protease inhibitor (SLPI) (15, 16). Although SLPI from cervical mucosal secretions has been shown to inhibit the pathogenesis of another bacterial sexually transmitted infection (STI), *Neisseria gonorrhoeae*, it does not seem to have an effect on *C. trachomatis* infectivity (17, 18).

Within the host cell there are cell autonomous responses that limit bacterial replication. In order to limit replication of a pathogen within a vacuole, such as *C. trachomatis,* the host must first recognize the existence of a pathogen-containing vacuole as a "non-self" entity. In murine epithelial cells, interferon-γ (IFNγ) signaling induces the expression of a family of IFNγ-inducible p47 guanosine triphosphate phosphatases (GTPases) termed immunity-related GTPases (IRGs) (19). The GTPases Irgm1 and Irgm3 recognize and decorate "self" vacuoles within the host cell, thus the absence of Irgm1 and Irgm3 from vacuoles is a signal to the host that the vacuole is pathogen-containing (20). Vacuoles that lack Irgm1/m3 become targets for other IRG proteins as well as Guanylate Binding Proteins (GBPs). IRG proteins can directly disrupt pathogen-containing vacuoles, releasing the contents of the vacuole into the cytosol where they can be targeted by autophagic processes (21). GBPs recruit additional antimicrobial factors including the NADPH oxidase NOX2, which produces potent anti-microbial reactive oxygen species anions (22, 23). Although *Chlamydia muridarum*, which has a specific tropism for mice, has evolved unknown mechanisms to evade restriction by the IRGs, *C. trachomatis* is extremely susceptible. Irgm1 and Irgm3 are required for murine-cellular

resistance to *C. trachomatis,* and mice lacking Irgm1/m3 exhibit delayed clearance of *C. trachomatis* (24).

Interestingly, humans lack the majority of the genes encoding the IRGs, and cell autonomous resistance to *C. trachomatis* in humans is mediated by a completely different mechanism (25). IFNγ signaling in human cells induces the expression of indoleamine-2,3-deoxygenase (IDO). IDO catabolizes the amino acid tryptophan and deprives *C. trachomatis* of this essential nutrient. Upon tryptophan starvation, *C. trachomatis* enters into a non-replicative persistent state (26). However, *C. trachomatis* expresses a tryptophan synthase, which is an enzyme capable of synthesizing tryptophan from indole (27). This persistent state is reversible if either tryptophan or indole becomes available (26, 27). It is believed that *C. trachomatis* can overcome tryptophan starvation in the genital tract by using indole produced from the vaginal flora (19). Interestingly, *C. muridarum* lacks a functional tryptophan synthase, suggesting that *Chlamydia* species have separately evolved host-specific immune evasion mechanisms.

Although it is not completely clear how, *C. trachomatis* can bypass the innate barriers of the lower genital tract and ascend to and establish infection in the upper genital tract. The epithelial cells that line the uterus produce an array of antimicrobial molecules and cytokines that are meant to create a pathogen-restrictive inflammatory environment. The defensins, HD5 and HNP2 are upregulated during infection with *C. trachomatis* and HNP2 is able to inhibit *C. trachomatis* infectivity, likely by permeabilizing the EB membrane (28, 29). It has also been shown that cultured cervical epithelial cells secrete the inflammatory cytokines interleukin-8 (IL-8), IL-6, tumor necrosis factor-α (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-

CSF) during *C. trachomatis* infection (30, 31). Additionally, it was shown that infected epithelial cells secrete IL-1α following lysis (30). These cytokines likely have a role in recruiting the innate immune cells, including natural killer (NK) cells, macrophages, neutrophils and dendritic cells. These innate immune cells can engulf and degrade extracellular EBs, as well as produce inflammatory cytokines such as IFNγ and TNFα, which restrict *C. trachomatis* growth in epithelial cells (32). Even though there is an early influx of innate immune cells to the upper genital tract upon *C. trachomatis* invasion, the innate response is not sufficient to limit infection and thus perhaps the more important role of the innate immune cells is to recruit and activate the cells of the adaptive immune response.

Adaptive Immune Responses to *C. trachomatis*

The adaptive immune response is absolutely essential for limiting *C. trachomatis* infection and providing a level of protection against reinfection. There are two main arms of the adaptive immune response: the humoral response mediated by B cells, and the cellular response mediated by $CD4^+$ and $CD8^+$ T cells. The activation of the adaptive immune response is believed to occur mainly within the lymph nodes that drain the genital tract, but there is also evidence of immune inductive sites within the genital tract (33, 34). In other mucosal tissues, such as the intestines, there are organized lymphoid structures in which immune cells reside and can be activated upon infection. However, these organized structures do not exist in the genital tract and instead there are clusters of immune cells that form the immune inductive sites (35). Dendritic cells actually provide the essential link between the innate and adaptive responses. After engulfing

extracellular EBs or infected epithelial cells, dendritic cells can present *Chlamydia* antigen to T cells either in the draining lymph nodes or immune inductive sites (35, 36).

Once activated, $CD4^+$ helper T cells can present antigen and activate B cells. The main role of B cells is to produce antibody to block pathogen entry into cells (37). There was early evidence suggesting that B cells might be an important mediator in limiting *C. trachomatis* infection. Epidemiological studies indicated an inverse correlation between the amount of *C. trachomatis* recovered from the cervix and the amount of *C. trachomatis* specific IgA antibody (38). However, subsequent studies demonstrated that mice lacking B cells did not exhibit exacerbated *C. trachomatis* infections (39). In fact there are current attempts to stimulate a protective B cell response. The dominant *C. trachomatis* antigen, major outer membrane protein (MOMP) is the primary candidate for a subunit vaccine and the main target to induce a humoral response. However, studies thus far have shown that there is extreme heterogeneity in MOMP sequences between the 16 *Chlamydia* serovars, which is impeding the development of a pan-serovar protective antibody (40).

Although the role of B cells during *C. trachomatis* infection is still debated, the importance of $CD4^+$ T cells is well described. Antigen presenting cells (APCs) that have engulfed extracellular *C. trachomatis* antigen can process this antigen and present the resulting peptides on major histocompatibility (MHC) class II molecules to stimulate naïve $CD4^+$ T cells. Activated $CD4^+$ T cells migrate to the site of infection and restrict *C. trachomatis* growth primarily through the secretion of the cytokine IFNγ. IFNγ induces the expression of IDO in humans, and IRGs in mice, to restrict *C. trachomatis* growth (described above). $CD4^+$ T cells are necessary in controlling *C. trachomatis* infection, as

mice that are depleted of $CD4^+$ T cells have higher bacterial levels and prolonged infections (41). Additionally, *C. trachomatis* specific $CD4^+$ T cells that are transferred into naïve mice are sufficient to provide mice with protection against infection (24, 41).

For many intracellular pathogens, $CD8^+$ T cells can be the main mediator of adaptive immunity. Unlike $CD4^+$ T cells that recognize extracellularly derived antigen, $CD8^+$ T cells recognize antigen that has been derived intracellularly. During *C. trachomatis* infection, dendritic cells can engulf EBs or infected epithelial cells and through a mechanism known as cross-presentation, present processed peptides on MHC class I molecules to activate $CD8^+$ T cells. Activated $CD8^+$ T cells proliferate and migrate to the genital tract where they are exposed to infected epithelial cells. During infection of epithelial cells, *C. trachomatis* uses the type III secretion system to inject proteins directly into the host cytosol. The MHC class I machinery can access and degrade the type III secreted effectors, as well as other proteins present on the inclusion membrane, and present the peptides to activated $CD8^+$ T cells. $CD8^+$ T cells that recognize infected cells can directly kill these cells through the secretion of perforin, which disrupts the epithelial cell membrane, and TNFα, which causes epithelial cells to undergo apoptosis. $CD8^+$ T cells can also secrete IFNγ to aid in restricting *C. trachomatis* infection.

The *C. trachomatis* antigen, CrpA, is the dominant epitope recognized by $CD8^+$ T cells (42). CrpA specific $CD8^+$ T cells have been shown to migrate to the uterus and proliferate in response to *C. trachomatis* infection (43). Additionally, naïve mice that receive activated CrpA specific $CD8^+$ T cells, derived from mice engineered to express only the T cell receptor specific for CrpA, are protected against subsequent *C.*

trachomatis infection (43). In fact, mice immunized against CrpA either through prior infection with Vaccinia virus expressing CrpA (Vac-CrpA) or *Listeria monocytogenes* expressing CrpA (*L.m.*-CrpA) are protected against subsequent systemic challenge with *C. trachomatis*. Although it is clear that $CD8^+$ T cells can protect against *C. trachomatis* infection, there is also evidence to suggest that the endogenous $CD8^+$ T cells provide little protection during natural infection. Unlike the protection generated by Vac-CrpA or *L.m.*-CrpA, naïve mice that receive $CD8^+$ T cells from mice previously infected with *C. trachomatis* are not protected from infection (42). Together these data indicate a perplexing role of $CD8^+$ T cells during infection.

Immune Memory Development

The cornerstone of immunity is the development of immunological memory such that hosts can quickly limit disease and clear pathogens that have been previously encountered. Epidemiological evidence indicates that people who have been previously infected with *C trachomatis* are still susceptible to subsequent *C. trachomatis* infection and disease sequelea, thus supporting the hypothesis that the immunological memory that develops during *C. trachomatis* infections is defective.

During primary *C. trachomatis* infection in the genital tract, $CD4^+$ T cells are primed in the draining lymph nodes (dLNs). Priming is the initial stage of T cell activation and sets the course for memory development. During $CD4^+$ T cell priming, the T Cell Receptor (TCR) on the $CD4^+$ T cell binds the antigen presented on MHC Class II by a professional antigen presenting cell (pAPC). In addition, co-stimulatory signaling by the pAPC is necessary to enhance TCR signaling in the T cell. Finally the third signal

needed for efficient priming and activation comes from cytokines, most predominantly IL-2, secreted both from the $CD4^+$ T cells and the pAPCs (44). Fully activated $CD4^+$ T cells migrate to peripheral tissues where they can be stimulated to express high levels of cytokines by APCs, despite no or low levels of costimulatory signals in those tissues. In the context of *C. trachomatis* infection, $CD4^+$ T cells migrate to the genital tract and expand over 30 fold to produce populations of $CD4^+$ T cells that can restrict *C. trachomatis* replication through the production of IFNγ (45, 46). The populations of $CD4^+$ T cells contract to form a stable pool of memory cells. $CD4^+$ T cells can develop into several types of memory cells: Th1, Th2, Th17 and T regulatory cells. The type of memory cell that develops is largely determined by the cytokines present in the milieu during priming. During *C. trachomatis* infection the main cytokines present are IFNγ and IL-12 (47, 48). These cytokines drive the $CD4^+$ T cells to develop into Th1 cells, which are potent producers of IFNγ. Studies have shown that mice that receive *Chlamydia*-specific Th1 skewed $CD4^+$ T cells are protected from infection with *C. trachomatis* (45). However mice that receive *Chlamydia*-specific Th2 cells, which predominantly produce the cytokine IL-4, actually have higher bacterial levels (45).

Memory $CD4^+$ T cells are defined by the retention of the Th1/Th2 cytokine profile, rapid production of cytokines, and the ability to be restimulated with lower antigen dose. Ultimately it is these characteristics that allow rapid pathogen clearance upon re-exposure. The memory Th1 cells that develop after primary *C. trachomatis* infection have the ability to rapidly expand upon reinfection and are superior at producing multiple cytokines IL-2, IFNγ, and TNFα compared to $CD4^+$ T cells generated during primary infection (49). In fact $CD4^+$ T cells are absolutely required for protection

against secondary *C. trachomatis* infection, as depletion of memory $CD4^+$ T cells prior to secondary challenge in mice results in high bacterial levels (41). Additionally, these memory $CD4^+$ T cells alone are sufficient to confer protection in naïve mice. Mice that receive memory $CD4^+$ T cells from previously infected mice are subsequently protected against *C. trachomatis* infection (Georg Stary, unpublished). Collectively these data support that a robust and efficient memory $CD4^+$ T cell response develops following *C. trachomatis* infection.

Like $CD4^+$ T cells, the development of memory $CD8^+$ T cells begins during priming. During *C. trachomatis* infection $CD8^+$ T cells are primed in the dLNs by recognizing antigen presented on MHC class I of dendritic cells. In addition to antigen stimulation through the TCR, $CD8^+$ T cells require costimulatory signaling mediated by CD28 expressed on the $CD8^+$ T cell membrane and CD80 or CD86 expressed on dendritic cells. Finally, $CD8^+$ T cells require a third signal of inflammatory cytokines, typically IFNγ and IL-12, to become fully activated. Activated $CD8^+$ T cells, subsequently termed effector $CD8^+$ T cells, can expand in number and migrate to the site of infection. Effector $CD8^+$ T cells recognize cognate antigen presented on infected cells and produce the lytic cytokines to kill those cells. Similar to $CD4^+$ T cells, the $CD8^+$ T cell population contracts several days after initial expansion and about 90% of the activated $CD8^+$ T cells are eliminated. What remains is a pool of memory $CD8^+$ T cells (**Figure 1-2**).

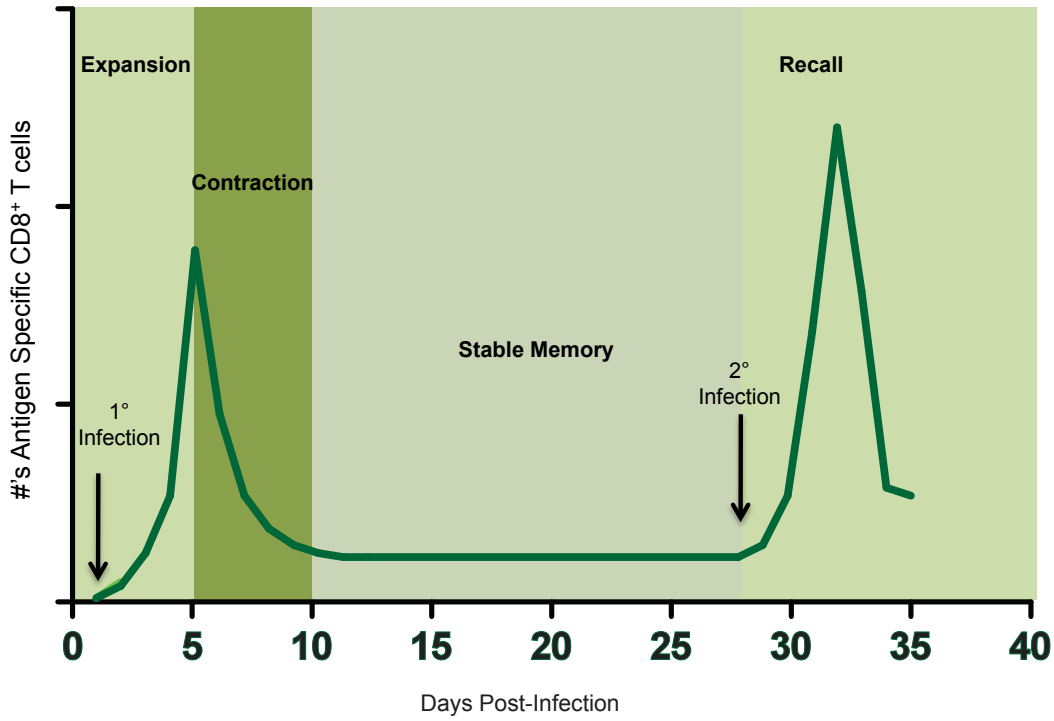

Figure 1-2 CD8$^+$ T cell response directed against pathogen challenge. Upon primary challenge (first arrow) with a pathogen, the CD8$^+$ T cells expand in number. After the peak of expansion, 90% of the CD8$^+$ T cells are deleted and the remaining population forms the memory pool. When the same antigen is encountered again (second arrow), the CD8$^+$ T cells respond more quickly and expand to higher numbers compared to primary response. The pathogen is quickly cleared, significantly limiting disease.

After the peak of CD8$^+$ T cell expansion and subsequent contraction of this population, the remaining 10% of CD8$^+$ T cells are retained as a pool of memory cells. Like memory CD4$^+$ T cells, the purpose of memory CD8$^+$ T cells is to provide a population of cells that can quickly respond when a pathogen is re-encountered. Functional memory CD8$^+$ T cells have a lower threshold of activation and can more readily produce cytokines such as IFNγ and TNFα. The quick response of memory CD8$^+$ T cells serves to limit pathogen replication in the host before it can cause disease. As mentioned, the development of the memory CD8$^+$ T cells depends largely on factors that

occurred during priming, as well as environmental cues. The three signals described above, TCR signaling, costimulatory and inflammatory cytokines, are sufficient for driving a robust primary $CD8^+$ T cell response. However, these signals are not sufficient for developing stable memory $CD8^+$ T cells. $CD4^+$ T cells are also necessary during the priming of $CD8^+$ T cells to promote the development of stable $CD8^+$ memory T cells that can elicit robust secondary responses. The primary function of "CD4 help" is to promote IL-2 signaling. IL-2 inhibits the cell death signaling pathway that is induced in $CD8^+$ T cells that have been repeatedly stimulated and undergone extensive expansion (50, 51). Current data suggest that $CD4^+$ T cell help can be indirect. CD40L expressed on $CD4^+$ T cells signals through CD40 on dendritic cells to activate these dendritic cells (52). Activated dendritic cells are then able to interact with $CD8^+$ T cells to prime the $CD8^+$ T cells to produce their own IL-2 (53). Therefore, $CD8^+$ T cells must be able to produce and respond to IL-2 in order to develop into functional memory cells (53).

$CD8^+$ T cells differentiate into subsets of memory cells, defined by several characteristics, which determine the type of secondary response produced. The majority of memory $CD8^+$ T cells express the IL-7 receptor (IL-7R) and the IL-15 receptor (IL-15R). Both IL-7 and IL-15 are required for memory $CD8^+$ T cells to maintain a basal level of proliferation, also termed homeostatic turnover (54, 55). Factors such as antigen load, inflammation, and cytokine signaling influence the type of memory $CD8^+$ T cell that develops. A subset of $CD8^+$ memory cells maintains high expression levels of the surface molecule CD62L. CD62L sustains T cell rolling in high endothelial venules (HEV), which supports reentry of T cells into lymphoid tissue (56). Cells expressing

high levels of CD62L are known as central memory $CD8^+$ T cells (T_{cm}). Alternatively, effector memory $CD8^+$ T cells (T_{em}) shed surface CD62L (57).

Effector and central memory $CD8^+$ T cells differ in their ability to expand, respond to antigen and migrate to the sites of infection. T_{cm} cells are primarily found in secondary lymphoid organs, as well as the spleen and blood and have an enhanced ability to expand with a low threshold of antigen stimulation. Thus, for systemic infections with pathogens such as Lymphocytic Choriomeningitis Virus (LCMV) Armstrong, Influenza, or *L. monocytogenes,* the T_{cm} is the major protective population of $CD8^+$ T cells (57). Effector memory T cells on the other hand typically circulate between the blood and peripheral tissues, and thus are believed to have enhanced access to peripheral infection. It has also been demonstrated that T_{em} cells have a higher capacity to produce effector cytokines, such as IFNγ and TNFα, and can be stimulated by nonprofessional APCs, but have lower expansion compared to T_{cm} cells (58, 59). During cutaneous infection with Herpes Simplex Virus (HSV), the T_{em} population is required for protection (60). The T_{em} population is also required for protection against certain viruses that infect the lung, indicating that this population can be a protective, especially at mucosal surfaces, despite the low expansion capacity of these cells (61).

Interestingly the development of T_{cm} versus T_{em} is still unclear. It has been well documented that over time the T_{cm} population in mice increases substantially over the T_{em} population . There is data that suggests a linear development in which T_{em} cells eventually develop into T_{cm} cells over time. In this model, T_{em} cells represent a transitional phase of $CD8^+$ T cells that overtime re-express CD62L (58, 62, 63). However, another model suggests that T_{cm} and T_{em} cells are distinct populations, and the

increase in T_{cm} cells over time is an indication of their enhanced expansion capability over T_{em} cells (64, 65). There are also discrepancies in the literature on the exact definition of T_{em} cells. T_{em} were traditionally defined as the "peripheral" memory $CD8^+$ T cells because they were the primary memory population found in peripheral tissue. However, evidence suggesting that T_{em} cells circulate between the periphery and blood has brought up questions of whether T_{em} populations reside in peripheral tissues or are transient visitors. Thus, an additional subset of memory $CD8^+$ T cells, termed resident memory (T_{rm}), were described as $CD8^+$ T cells that permanently reside in specific tissues and do not re-circulate (66).

T_{rm} cells were traditionally defined by the expression of the CD103 molecule. CD103 functions as a receptor for E-cadherin, an adhesion molecule specifically expressed by epithelial cells (67). T_{rm} cells have been described for the skin, intestinal mucosa, brain and the vaginal mucosa (68). Vaginal infection with the Human Papillomavirus (HPV) stimulates the expansion and retention of $CD103^+$ $CD8^+$ T cells within the vaginal mucosa. Upon vaginal rechallenge these $CD8^+$ T cell provide superior protection compared to systemic $CD8^+$ T cells (69). Ultimately, it remains unclear which cellular $CD8^+$ population mediates protection for the upper genital tract.

$CD8^+$ T cell dysfunction during infection

The $CD8^+$ T cell response is a critical component of the immune response to intracellular pathogens. However, a $CD8^+$ T cell response that is too robust can cause severe damage to host tissue, thus there are host mechanisms in place to limit $CD8^+$ T cell expansion and cytokine production. These inhibitory mechanisms are important for

maintaining peripheral tolerance and protecting the host from autoimmunity and pathology. During activation, $CD8^+$ T cells express a variety of costimulatory and inhibitory receptors and ligands. This provides a combination of signaling patterns that influence the expression of cytokines and proliferative responses, determining the activation and effector state of the $CD8^+$ T cell. During an interaction with a pAPC, the engagement of immune-inhibitory molecules can reduce the signaling that originates from the TCR and costimulatory receptors. For example, the molecule PD-L1 expressed by a pAPC engages PD-1 expressed on a $CD8^+$ T cell (70, 71). This interaction causes the dephosphorylation PI3K, disrupting this signaling pathway and subsequently limiting the expression of IFNγ, IL-2 and the cell-survival molecule Bcl-xL (72) (**Figure 1-3**).

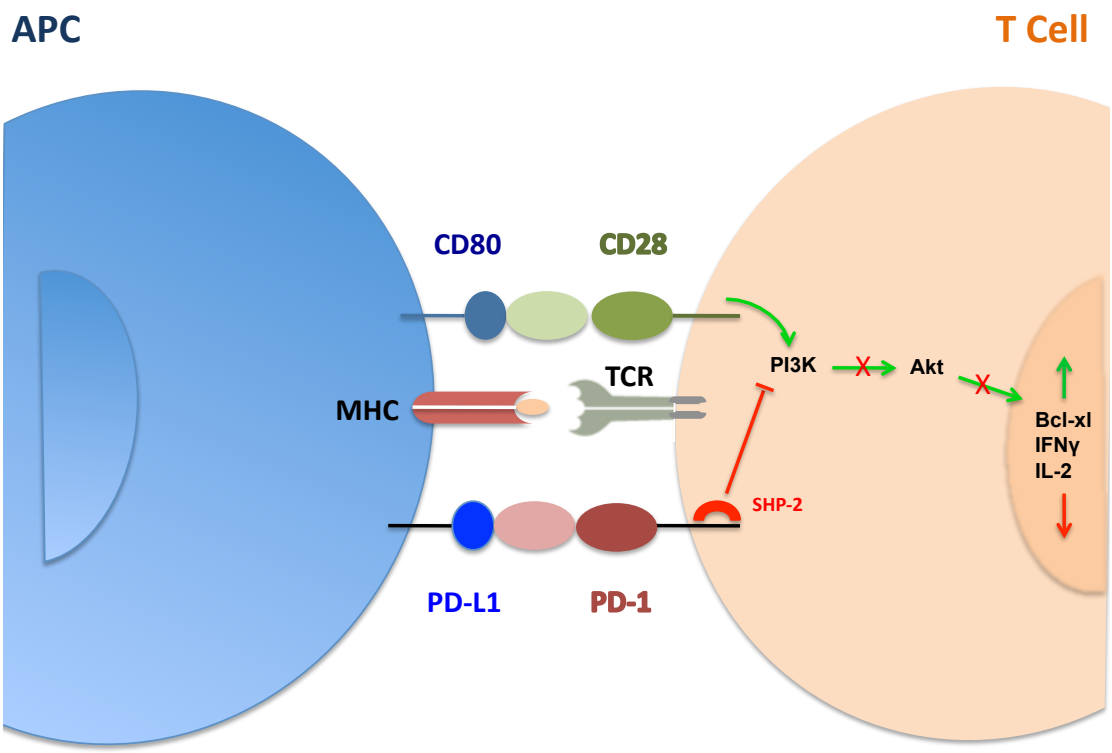

Figure 1-3 PD-1 dampens TCR signaling. During interaction with an APC, the T cell response is mediated by costimulatory (green signals) and inhibitory (red signals) molecules. When PD-1 is engaged by PD-L1, the SHP-2 phosphatase is recruited and prevents signaling from the costimulatory molecule CD28. Adapted from (73).

The engagement of immune-inhibitory molecules, such as PD-1, does not just affect that immediate $CD8^+$ T cell response. PD-1 signaling during priming of the $CD8^+$ T cell can permanently alter the $CD8^+$ T cell. $CD8^+$ T cells that express PD-1 and are engaged by PD-L1 become exhausted and this has long-term consequences. Exhausted $CD8^+$ T cells progressively lose the ability to produce IFNγ, TNFα and IL-2. Exhausted $CD8^+$ T cells also fail to acquire key memory properties such as homeostatic turnover and the ability to mount robust recall responses. Eventually, exhausted $CD8^+$ T cells undergo

apoptosis, and in the context of infection this can substantially reduce the antigen-specific $CD8^+$ T cell population (74).

A number of pathogens have evolved mechanisms to exploit the PD-1/PD-L1 pathway to evade the $CD8^+$ T cell response and subsequently cause persistent infection. For example, during Lymphocytic Choriomeningitis Virus clone-13 (LCMV-Cl13) infection, PD-1 expressing $CD8^+$ T cells become exhausted and are unable to clear the virus, thereby contributing to chronicity of the infection (75). However, blockade of the PD-1/PD-L1 pathway through antibody treatment can reverse $CD8^+$ T cell dysfunction and restore viral clearance (76).

Similar to LCVM-Cl13, Human Immunodeficiency Virus (HIV) infection results in the expression of PD-1 on HIV specific $CD8^+$ T cells and the subsequent exhaustion of these $CD8^+$ T cells. Studies of PD-1 expressing HIV specific $CD8^+$ T cells from human patients found that PD-1 expression positively correlated with the expression of the transcription factor basic leucine zipper transcription factor ATF-like (BATF) (77). Additionally, the engagement of PD-1 with PD-L1 led to the increase in BATF expression. Over-expression of BATF in $CD8^+$ T cells led to increased apoptosis and reduced IL-2 production (77). These studies have provided mechanistic insight into how PD-1 results in $CD8^+$ T cell dysfunction.

$CD8^+$ T cell impairment during *C. trachomatis* infection

Like many chronic infections, *C. trachomatis* infection produces a $CD8^+$ T cell response that is impaired. During primary systemic infection with *C. trachomatis* there is actually a very robust $CD8^+$ T cell response. However, as described previously, the

hallmark of immune memory is the ability to respond more quickly to rechallenge. Upon systemic rechallenge with *C. trachomatis,* there is an extremely blunted secondary $CD8^+$ T cell response (78). For many chronic infections, sustained antigen exposure is what drives $CD8^+$ T cell impairment. However, for *C. trachomatis* this does not seem to be the case as mice treated with doxycycline to ensure complete *C. trachomatis* clearance still exhibit a blunted recall response (78).

The mechanism behind the blunted $CD8^+$ T cell response during *C. trachomatis* reinfection remained unclear. However, it appeared that *C. trachomatis* infection resulted in a global suppression of $CD8^+$ T cells. This was demonstrated by co-infection with *C. trachomatis* and *Listeria monocytogenes.* Mice that were infected previously or simultaneously with *C. trachomatis* exhibited a reduced $CD8^+$ T cell response to *L. monocytogenes,* compared to mice that were only infected with *L. monocytogenes* (78). These results confirmed that *C. trachomatis* infection can alter the $CD8^+$ T cell response to a heterologous antigen, suggesting that *C. trachomatis* infection elicits a broad inhibitory mechanism. Additionally these studies provided the key evidence to understanding why natural immunity to *C. trachomatis* is ineffective at preventing subsequent infection.

Vaccine development against *C. trachomatis*

Studies of humans and mice have indicated that natural immunity towards *C. trachomatis* is impaired and fails to protect the host against reinfection. Thus, having a vaccine that can produce superior protection is of the upmost importance. In general vaccines can be DNA, subunit or cellular based. The majority of vaccine studies for *C.*

trachomatis have focused on subunit based immunizations. As described earlier, the *C. trachomatis* protein MOMP is a major B cell antigen. MOMP contains four variable domains that are antigenically variable among the 16 serovars of *C. trachomatis* and have made it difficult to induce pan-serovar protection. However, recent studies have shown that immunization with recombinant MOMP (rMOMP) elicits antibody and $CD4^+$ T cell responses specific for constant domains of MOMP (79). Immunization with rMOMP resulted in protection against several other *C. trachomatis* serovars, demonstrating that it is possible to generate heterotypic immunity against MOMP.

Another antigen at the forefront of vaccine studies is the chlamydial protease-like activity factor (CPAF). CPAF is expressed in *C. trachomatis* RBs, secreted out of the inclusion and predominantly found in the host cytosol. Mice immunized with recombinant CPAF produce a $CD4^+$ T cell response that is protective against vaginal challenge with *C. muridarum* (80).

Interestingly, less attention has been paid to investigating a protective $CD8^+$ T cell response. This is likely due to the abundance of data demonstrating that memory $CD8^+$ T cells are impaired and offer little protection in the context of a natural infection. However, as described earlier, immunization against the $CD8^+$ T cell antigen, CrpA, can protect mice against subsequent systemic infection. In general, $CD8^+$ T cells are the sentinels against intracellular pathogens and it is $CD8^+$ T cells that can directly kill infected cells. Presumably, creating a combinatorial vaccine that elicits an antibody and a protective $CD4^+$ and $CD8^+$ cellular response will be most effective.

Several important caveats exist with current *Chlamydia* vaccine studies. First, there is variability in the species used during studies. Identifying antigens that protect

against the mouse pathogen, *C. muridarum,* cannot necessarily be extrapolated to the human pathogen *C. trachomatis*. More important is the variability in the site of immunization and the site of challenge. Studies have used different routes of immunization that produce phenotypically different cellular responses. Ultimately, to produce a protective response it will be important to immunize by a route that generates immune cells that can quickly migrate to the genital tract. Attention should be paid to the HSV literature. Researchers have described a $CD8^+$ T cell population that resides in the genital skin and mucosa that provides protection against subsequent HSV challenge (81). Additional studies have identified a method termed "prime and pull" for generating local $CD4^+$ and $CD8^+$ T cell memory in the vaginal mucosa. In the prime and pull method, mice were subcutaneously immunized (or primed) with an attenuated HSV strain. Subsequently, chemokines were topically applied to the vaginal cavity of immunized mice to "pull" the $CD4^+$ and $CD8^+$ T cells to the genital tract. The $CD4^+$ and $CD8^+$ T cells remained in the vaginal mucosa long term and provided superior protection compared to mice that did not receive the "pull" (82). Whether the prime and pull method will work for other genital pathogens has not been investigated.

The HSV literature provides promising avenues for generating a protective vaccine against *C. trachomatis*. Understanding how to generate local immunity will be critical to designing an effective vaccine against *C. trachomatis.* However, significant differences exist between the two pathogens. Most significant, in terms of generating an immune response, is the fact that in humans *C. trachomatis* does not stay in the vaginal cavity but ascends to the upper genital tract. The immune responses within the vaginal cavity and upper genital tract differ. Recent studies have shown that mice intravaginally

infected with *C. trachomatis,* do not produce a significant $CD4^+$ T cell response compared to mice that have been inoculated with *C. trachomatis* directly into the uterus (41). It is likely that $CD8^+$ T cell responses differ as well, although this has not been investigated. Again these data point to the caveats of vaccine studies against *C. trachomatis* and the need to be aware of the location of bacterial challenge. Ideally, future vaccine studies will investigate responses to *C. trachomatis* within the upper genital tract.

Focus of dissertation

C. trachomatis should be highly susceptible to the $CD8^+$ T cell response. However, the natural $CD8^+$ T cell response that is elicited upon *C. trachomatis* infection appears to play an insignificant role in protecting the host. Moreover, the $CD8^+$ T cell response is highly impaired and *C. trachomatis* can actually dampen the $CD8^+$ T cell response to other pathogens which has significant implications for hosts that may be coinfected with *C. trachomatis* and any other genital pathogen. I began my studies to more fully understand the endogenous $CD8^+$ T cell response to *C. trachomatis* infection of the genital tract. Although the *C. trachomatis* specific $CD8^+$ T cell response had previously been described, those studies were performed using systemic infection and it remained unclear how the mucosal $CD8^+$ T cell response functioned. What I have learned studying the response in the genital tract complements what has been described for systemic infection. Furthermore, my studies led me to investigate mechanisms that impair the $CD8^+$ T cell response in the genital tract during *C. trachomatis* infection. I

discovered that the PD-1/PD-L1 pathway alters the $CD8^+$ T cell response and contributes to limiting *C. trachomatis* clearance.

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Chapter Two: The $CD8^+$ T cell response to *C. trachomatis* infection of the genital tract is impaired.

PD-L1 limits the mucosal CD8$^+$ T cell response to *Chlamydia trachomatis*

Sarah Fankhauser and Michael Starnbach

Portions of this chapter have been submitted as part of a manuscript to the Journal of Immunology. The project was conceived by S. Fankhauser and M. Starnbach. Experiments and data analysis were performed by S. Fankhauser, and all text and figures were produced by S. Fankhauser.

Introduction

Infection with *Chlamydia trachomatis* stimulates both the innate and adaptive immune responses (1). A clear understanding of the adaptive immune response to *C. trachomatis* will be key in developing a protective vaccine. Studies have demonstrated that $CD4^+$ T cells are both necessary and sufficient to protect mice against *C. trachomatis* in the genital tract of mice (2). However, because of the obligate intracellular nature of *C. trachomatis* there is interest in understanding the role that the $CD8^+$ T cell response may play in clearance of *C. trachomatis.* $CD8^+$ T cells are generally stimulated by cytosolic derived antigen presented on Major Histocompatibility Complex class I (MHC class I), although professional antigen presenting cells (pAPCs) can take up extracellular antigen and present it on MHC class I through a process known as cross-presentation. In infected epithelial cells, *C. trachomatis* proteins that are inserted into the inclusion membrane or secreted outside of the inclusion are the most likely candidates to be processed and presented on MHC class I.

Previous research has demonstrated that $CD8^+$ T cells are efficiently primed during *C. trachomatis* infection (3, 4). To date, seven *C. trachomatis* $CD8^+$ T cell antigens have been identified that are stimulated upon *C. trachomatis* infection (5-7). Of these seven, one antigen, CrpA, has been best described (3, 6). It has been demonstrated that systemic infection with *C. trachomatis* stimulates a robust primary expansion of CrpA-specific $CD8^+$ T cells (8). As described in the Introduction, with acute pathogens such as Influenza or *Listeria monocytogenes*, the secondary $CD8^+$ T cell expansion upon rechallenge would be expected to occur more quickly and with a higher magnitude

compared to primary challenge (9, 10). However, during systemic rechallenge with *C. trachomatis,* the secondary CrpA-specific $CD8^+$ T cell expansion occurs with the same kinetics as primary expansion, with the peak of both primary and secondary $CD8^+$ T cell responses occurring at day seven-post infection. Furthermore, the peak of secondary is significantly reduced in number compared to the peak of the primary response (8). It was hypothesized that *C. trachomatis* systemic infection impairs the $CD8^+$ T cell response, and this was supported by experiments demonstrating that primary co-infection of mice with *C. trachomatis* and *L. monocytogenes* produce fewer *L. monocytogenes* specific $CD8^+$ T cells compared to mice infected with the same dose of *L. monocytogenes* alone (8). Thus not only does *C. trachomatis* infection impair the recall capacity of the $CD8^+$ T cell response, but these data suggest that *C. trachomatis* reduces the ability of the primary $CD8^+$ T cells to respond to heterologous challenge. Yet, how *C. trachomatis* infection inhibits the $CD8^+$ T cell response remained unclear.

Although the $CD8^+$ T cell recall response to *C. trachomatis* is impaired, it is possible to generate protective $CD8^+$ T cell responses against *C. trachomatis*. Mice immunized with Vaccinia expressing CrpA are protected against subsequent challenge with *C. trachomatis* (6). Additionally, mice that receive $CD8^+$ T cells that are engineered to specifically express the T Cell Receptor (TCR) for CrpA are protected against systemic infection with *C. trachomatis* compared to mice that receive no T cells (3). These experiments provide strong support to the hypothesis that $CD8^+$ T cells have the ability to be an important protective response against *C. trachomatis.*

A significant caveat to the studies described above is that these experiments were performed using a systemic model of infection. In humans, *C. trachomatis* infects

mucosal surfaces, specifically ocular and uterine epithelial cells, which generate phenotypically distinct immune responses compared to the systemic site of infection. For example, $CD8^+$ and $CD4^+$ T cells require a specific set of chemokines in order to migrate to the genital tract, that are not required for systemic infection (11, 12). It has previously been difficult to track the endogenous *C. trachomatis* specific $CD8^+$ T cell responses in the genital tract. However, retrogenic $CD8^+$ T cells specific for CrpA were shown to proliferate, produce IFNγ and migrate to the genital tract in response to intrauterine infection with *C. trachomatis* (3). What still remained unclear from previous research was the nature of the endogenous $CD8^+$ T cell response to genital infection with *C. trachomatis.* Specifically it was unknown whether the impaired $CD8^+$ T cell response observed during systemic infection also occurs in the genital tract of *C. trachomatis* infected mice. This chapter describes the endogenous $CD8^+$ T cell response to *C. trachomatis* infection of the murine genital tract and demonstrates that *C. trachomatis* infection of the genital tract produces an impaired $CD8^+$ T cell response that does not contribute to bacterial clearance.

Results

Characterizing the *C. trachomatis*-specific $CD8^+$ T cell response in the genital tract

Previous work has described the $CD8^+$ T cell response during a systemic *C. trachomatis* infection. However, little is known regarding the endogenous $CD8^+$ T cell response to *C. trachomatis* infection in the genital tract. To characterize the mucosal $CD8^+$ T cell response to *C. trachomatis,* I transcervically infected mice with 10^6 inclusion forming units (IFU) of *C. trachomatis*. At specific time points following primary infection, during memory phase and after secondary infection, I measured the number of *C. trachomatis* specific $CD8^+$ T cells in the draining lymph nodes (dLNs) and the uterus. Using an ELISPOT assay, I measured the number of IFNγ producing $CD8^+$ T cells specific for the *C. trachomatis* immunodominant antigen, CrpA, in the dLNs. Five days after infection, the number of IFNγ producing $CrpA^+$ T cell population had expanded 1,700 fold compared to day zero (**Figure 2-1**).

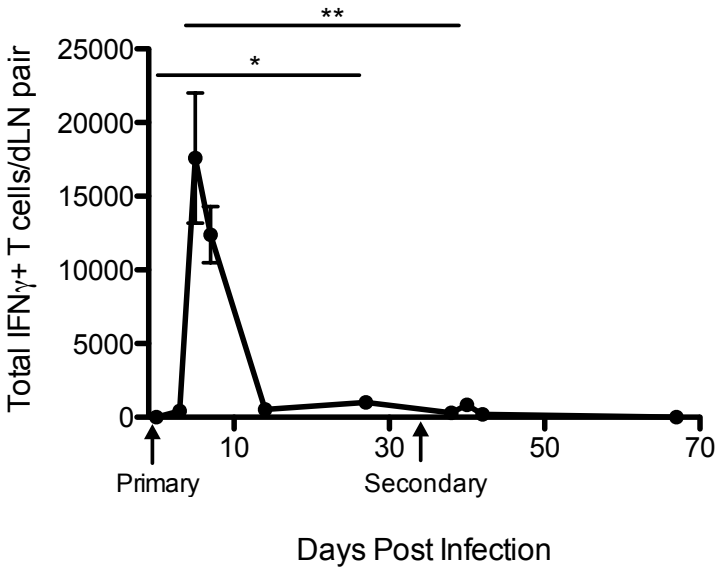

Figure 2-1 CrpA specific CD8$^+$ T cell response in the dLNs during transcervical *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis* on day 0 (marked by 1st arrow). At time points marked, dLNs were harvested and the number of CrpA specific IFNγ-producing CD8$^+$ T cells was measured by ELISPOT. Mice were rechallenged at the second arrow. Each time point is the mean of five mice per group, and bars represent SEM. Statistical significance of *$p<0.05$ is indicated for day 0 and day 28. **$p<0.01$ is indicated for day 5 primary and day 5 secondary infections. Statistics were determined by Mann-Whitney Test.

By day twenty-eight following infection, the number of IFNγ producing CrpA$^+$ T cells was substantially reduced compared to the peak of primary at day five, however there remained a memory population of CrpA T cells that was significantly higher in number compared to day zero (**Figure 2-1**).

Other work has demonstrated that the secondary CD8$^+$ T cell response to acute pathogens such as *L. monocytogenes* and LCMV should occur faster and with a higher magnitude compared to the primary CD8$^+$ T cell response to that same pathogen. Therefore I expected that mice rechallenged with *C. trachomatis* would produce a secondary CD8$^+$ T cell response that was more robust compared to the primary response

observed. Thirty-five days after primary infection, I transcervically rechallenged mice with 10^6 IFU of *C. trachomatis*. Compared to primary infection, there was not a robust expansion of the IFNγ producing $CrpA^+$ T cells. In fact, the peak of the secondary CrpA T cell response five days following secondary challenge, which should include both newly primed and antigen-experienced $CD8^+$ T cells, was 21 fold lower than the peak of the primary response (**Figure 2-1**). Thirty days after secondary challenge, the $IFN\gamma^+$ CrpA population had returned to a level similar to day zero. These data suggest that further antigen challenge caused the *C. trachomatis*-specific $CD8^+$ T cell population to either die or become unresponsive. All together these data demonstrate that *C. trachomatis* stimulates robust $CD8^+$ T cell expansion during primary infection, but is unable to elicit a comparable response during secondary infection.

Although there was a substantial $CD8^+$ T cell population in the dLNs upon primary infection, I questioned whether this translated to a *C. trachomatis*-specific $CD8^+$ T cell migration to the site of infection in the uterus. Using a previously described tetramer specific for the immunodominant *C. trachomatis* antigen, CrpA, I measured the endogenous $CD8^+$ T cell response in the genital tract by flow cytometry during primary infection, memory response, and secondary infection. The number of $CrpA^+$ $CD8^+$ T cells expanded over 1500 fold between days zero and seven in the genital tract (**Figure 2-2**). After the peak of expansion, which occurred seven days following infection, the $CrpA^+$ $CD8^+$ T cell population contracted. By day twenty-seven post infection the number of $CrpA^+$ $CD8^+$ T cells found in the genital tract was similar to day zero (**Figure 2-2**).

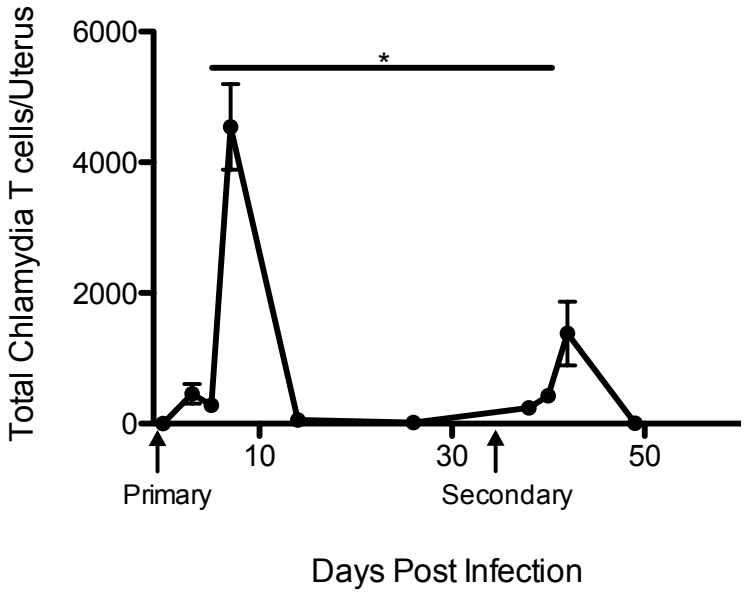

Figure 2-2 CrpA specific CD8$^+$ T cell response in the genital tract during transcervical *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis* on day 0 (marked by 1st arrow). At time points marked, genital tracts were harvested and the number of CD8$^+$ T cells positive for the CrpA tetramer was measured by flow cytometry. Mice where rechallenged at the second arrow. Each time point is the mean of five mice per group, and bars represent SEM. Statistical significance of *$p<0.05$ is indicated for day 7 primary and day 7 secondary infections by Mann-Whitney Test.

As shown in **Figure 2-1**, there were very few CrpA-specific CD8$^+$ T cells in the dLNs during secondary infection with *C. trachomatis.* I hypothesized that the low number of CD8+ T cells in the dLNs may be an indication that the activation and expansion of the secondary T cell response might occur in the uterus, and therefore a robust secondary response should be observed in the uterus during secondary infection. To test this, following the resolution of infection and the formation of a *C. trachomatis* specific memory CD8$^+$ T cell population, I transcervically rechallenged mice thirty-five days after the primary infection. The peak of the secondary CrpA$^+$ CD8$^+$ T cell response

in the genital tract was seven days following challenge, however the secondary response was substantially reduced compared to the primary infection with 3.5 fold fewer $CrpA^+$ T cells found at the peak of secondary infection compared to primary infection (**Figure 2-2**). Together these data demonstrate that there is a substantial expansion of the primary *C. trachomatis*-specific $CD8^+$ T cell population, however there is a severely blunted secondary response in both the dLNs and the uterus.

$CD8^+$ T cells are dispensable during *C. trachomatis* infection

The previous data suggests that there is an impaired secondary $CD8^+$ T cell response to *C. trachomatis.* However I questioned whether this correlated with a decrease in the number of effector $CD8^+$ T cells present in the genital tract following secondary infection. IFNγ is the main cytokine necessary to restrict to *C. trachomatis* replication in the uterus, therefore I measured the number of IFNγ-producing $CD8^+$ T cells present in the genital tracts by intracellular cytokine staining of mice after primary and secondary infections. Six days after secondary infection, there was a six-fold decrease in the number IFNγ-producing $CD8^+$ T cells compared to primary infection (**Figure 2-3a)**. Upon examining the mean fluorescent intensity of the IFNγ-producing population, it was evident that the $CD8^+$ T cells from both primary and secondary infections produced similar amounts of IFNγ (**Figure 2-3b)**. I further analyzed the total $CD8^+$ T cell population present in the uterus to examine if the decrease in secondary IFNγ-producing $CD8^+$ T cells was associated with an overall decrease in the $CD8^+$ T cell population. There was a two-fold decrease in the overall number of $CD8^+$ T cells in the uterus during secondary infection compared to primary (**Figure 2-3c**). However, there

was also a significant two-fold decrease in the percentage of uterine $CD8^+$ T cells producing IFNγ (**Figure 2-3d)**. These data demonstrate that the magnitude of the cytokine producing secondary $CD8^+$ T cell population is significantly reduced compared to the primary response to *C. trachomatis* infection. This reduction can not be fully attributed to an overall decrease in the number of $CD8^+$ T cells and suggests that IFNγ producing $CD8^+$ T cells are specifically deleted or impaired during secondary *C. trachomatis* infection.

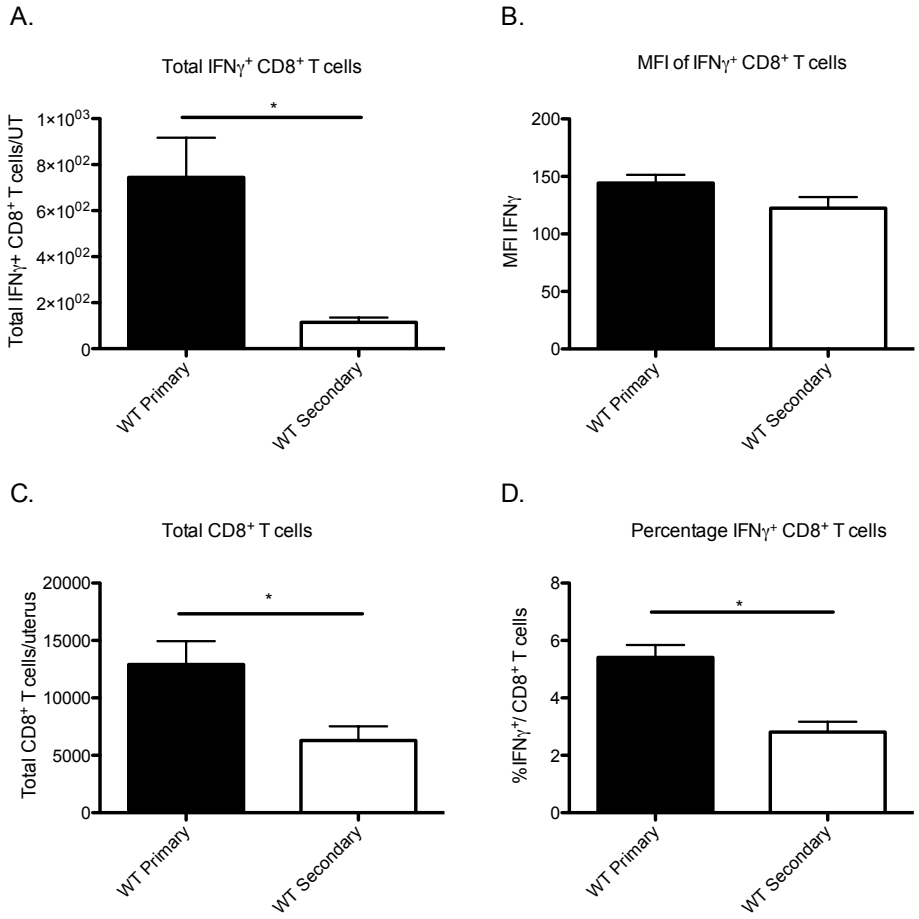

Figure 2-3. IFNγ response of uterine CD8$^+$ T cells 6 days post secondary infection is lower than primary infection. Mice were challenged with 10^6 IFU of *C. trachomatis.* A. Six days post primary or post secondary challenge, lymphocytes from the genital tract were stimulated and the number of IFNγ producing CD8$^+$ T cells were measured by ICCS. B. MFI was determined from IFNγ$^+$ CD8$^+$ T cell population. C. Total numbers of CD8$^+$ T cell were measured in the uterus by flow cytometry. D. The percentage of CD8$^+$ T cells that produce IFNγ. Bars indicate the mean of five mice per group and error bars represent SEM. *$p<0.05$ was determined by Mann-Whitney Test.

While it is clear that the $CD8^+$ T cell response is blunted during secondary infection, it remained unclear if the $CD8^+$ T cell population, during primary or secondary infection, contributed to *C. trachomatis* clearance. To investigate this, I treated mice with anti-CD8 depleting or isotype control antibody every day for three days prior to primary infection, and every other day after infection. I confirmed at least a 100-fold depletion of $CD8^+$ T cells in the uterus (**Figure 2-4a, left panel)** and observed similar depletion in the dLNs and spleens (data not shown). Five days post infection, which is the peak of *C. trachomatis* infection in the uterus, bacterial levels were measured by quantitative real-time polymerase chain reaction (qPCR). Mice depleted of $CD8^+$ T cells had similar bacterial levels in the uterus compared to mice treated with control antibody (**Figure 2-4a, right panel)**. On day seven when infection is beginning to be cleared from the uterus, both groups of mice still exhibited equivalent bacterial levels (**Figure 2-4b)**.

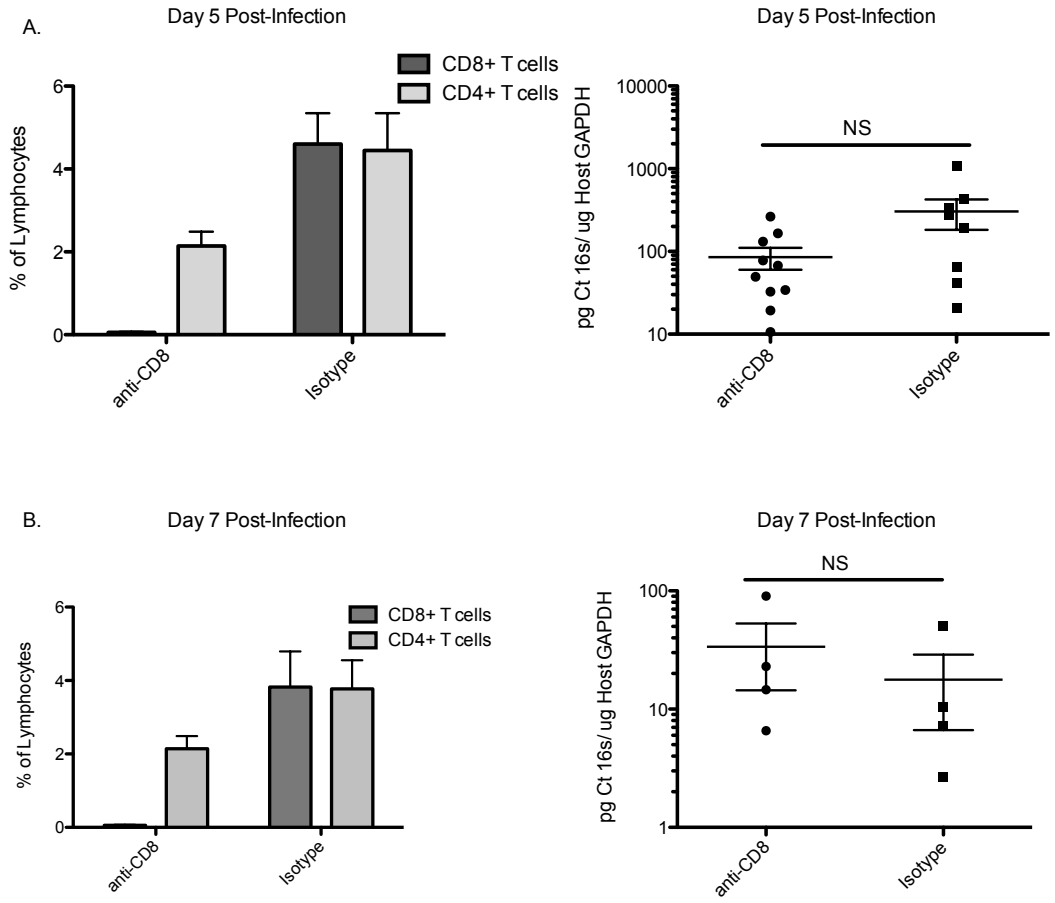

Figure 2-4 CD8$^+$ T cell depletion during *C. trachomatis* transcervical infection does not impact clearance. A. Mice were treated with anti-CD8 depleting antibody or isotype control 3 days prior to and every other day after primary transcervical infection with *C. trachomatis.* Five days post infection CD8$^+$ and CD4$^+$ T cell populations were measured in the uterus (left panel) and bacterial levels were measured by qPCR (right panel). B. Mice were treated with anti-CD8 depleting antibody or isotype control 3 days prior to and every other day after primary transcervical infection with *C. trachomatis.* Seven days post secondary infection CD8$^+$ and CD4$^+$ T cell populations were measured in the uterus (left panel), and bacterial levels were measured by qPCR (right panel). Bacterial levels were normalized to host GAPDH levels. Bars represent the mean of 4-9 mice per group, and error bars indicate SEM.

Although it appeared that CD8$^+$ T cells do not contribute to *C. trachomatis* clearance, it remained possible that the strong mouse-specific innate immune response

mediated by the Immunity Related GTPases (IRGs) masked a protective role for $CD8^+$ T cells (described in the Introduction). Previous work demonstrated that mouse IRGs are upregulated in response to IFNγ and can restrict *C. trachomatis* replication (13). However IRGs are not present in humans and do not contribute to *C. trachomatis* restriction in humans, thus deletion of mouse IRGs is believed to more closely recapitulate a human *C. trachomatis* infection. Mice deleted for the genes *Irgm1* and *Irgm3* (Irgm1/m3$^{(-/-)}$) show delayed but effective *C. trachomatis* clearance (14). However, Irgm1/m3$^{(-/-)}$ mice show an enhanced dependence on the adaptive immune response, specifically $CD4^+$ T cells, as depletion of $CD4^+$ T cells in these mice leads to significantly higher *C. trachomatis* levels (14). To test if the mouse innate immunity may mask a role for $CD8^+$ T cells, I treated Irgm1/m3$^{(-/-)}$ mice with $CD8^+$ depleting antibody or control antibody prior to primary *C. trachomatis* transcervical infection. Due to the delayed clearance defect of these mice I waited eleven days after infection to measure bacterial levels in the uterus. Both groups of mice showed equivalent bacterial levels. Together these data indicate that the $CD8^+$ T cell response is not necessary to control primary *C. trachomatis* infection and has no measurable impact on the clearance of *C. trachomatis* (**Figure 2-5)**.

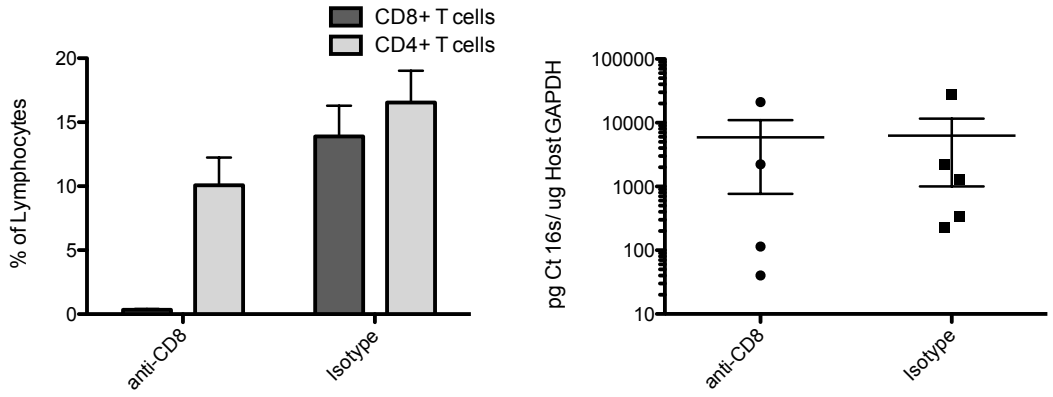

Figure 2-5 CD8$^+$ T cell depletion does not alter *C. trachomatis* levels during transcervical infection of Irgm1/m3$^{(-/-)}$ mice. Irgm1/m3$^{(-/-)}$ deficient mice were treated with anti-CD8 depleting antibody or isotype control three days prior to and every other day after primary transcervical infection with *C. trachomatis*. Eleven days post-infection, CD8$^+$ and CD4$^+$ T cell populations were measured in the uterus and shown as the percentage of CD90.2$^+$ lymphocytes (left graph). Bacterial levels were measured by qPCR and normalized to host GAPDH levels (right graph). Bars represent the mean of 4-5 mice per group and error bars indicate SEM.

It is clear that CD8$^+$ T cells do not contribute to clearance of primary *C. trachomatis* infection in the uterus. However, it remained a possibility that the memory CD8$^+$ T cells that developed after primary infection contributed to control of secondary infection. To test this, mice that recovered from primary infection were treated with anti-CD8 depleting antibody or isotype control. Five days after transcervical rechallenge, *C. trachomatis* levels in the uterus were measured by qPCR. Mice that were treated with anti-CD8 depleting antibody had bacterial levels similar to mice treated with isotype control (**Figure 2-6**). These data confirm that the secondary CD8$^+$ T cells do not contribute to control of *C. trachomatis* replication.

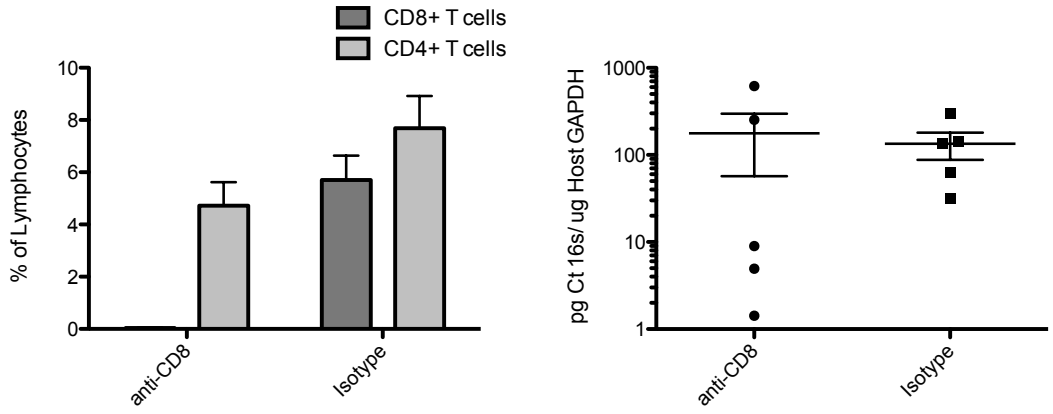

Figure 2-6 CD8$^+$ T cell depletion during secondary challenge does not impact *C. trachomatis* burden. Mice treated with anti-CD8 depleting antibody or isotype control 3 days prior to and 2 days after secondary transcervical infection with *C. trachomatis*. Five days post secondary infection CD8$^+$ and CD4$^+$ populations were measured in the genital tract and are shown as the % of CD90.2 lymphocytes (left graph). Bacterial levels were measured by qPCR and normalized to host GAPDH levels (right graph). Bars represent the mean of 5 mice per group and error bars indicate SEM.

Infection with *C. trachomatis* impairs the CD8$^+$ T cell response to *Listeria monocytogenes*

The data presented have clearly demonstrated that the CD8$^+$ T cell response that develops during a *C. trachomatis* infection 1. Exhibits a reduced secondary response that is numerically smaller compared to the primary response and 2. Does not contribute to control of *C. trachomatis*. I hypothesized that infection with *C. trachomatis* induces an immunosuppressive environment that results in mis-priming of the CD8$^+$ T cells and the impaired recall response. If this were true, then infection with *C. trachomatis* should also impair the CD8$^+$ T cell response to a heterologous antigen. Previous studies

demonstrated that systemic co-infection with *C. trachomatis* and *L. monocytogenes* resulted in a reduction in the number of *Listeria* specific $CD8^+$ T cells (8). I consequently decided to continue this system in the genital tract. Preliminary results demonstrated that a dose of 10^5 colony forming units (CFU) of *L. monocytogenes* deposited transcervically in mice produced a $CD8^+$ T cell response numerically comparable to 10^6 *C. trachomatis*, therefore this dose was continued for subsequent experiments. To test the hypothesis that *C. trachomatis* infection would impair the *Listeria* specific $CD8^+$ T cell response, I transcervically co-infected mice with 10^6 *C. trachomatis* and 10^5 *L. monocytogenes* engineered to express the Ova antigen (*L.m.*-Ova). Mice were either infected with *C. trachomatis* three hours prior to *L.m.*-Ova infection, or at the same time as *L.m.*-Ova infection, or infected with *L.m.*-Ova only (all mice received the same dose of *L.m.*-Ova). Five days after infection, I measured the numbers of Ova-specific $CD8^+$ T cells in the dLNs by ELISPOT. Mice that were co-infected with *C. trachomatis,* either three hours prior to or at the same time as *L.m.*-Ova infection, had significantly fewer Ova specific IFNγ producing $CD8^+$ T cells compared to mice infected with *L.m.*-Ova alone (**Figure 2-7a**). This was not due to differences in *L.m.*-Ova bacterial levels, as all mice had equivalent levels of bacteria (**Figure 2-7b**).

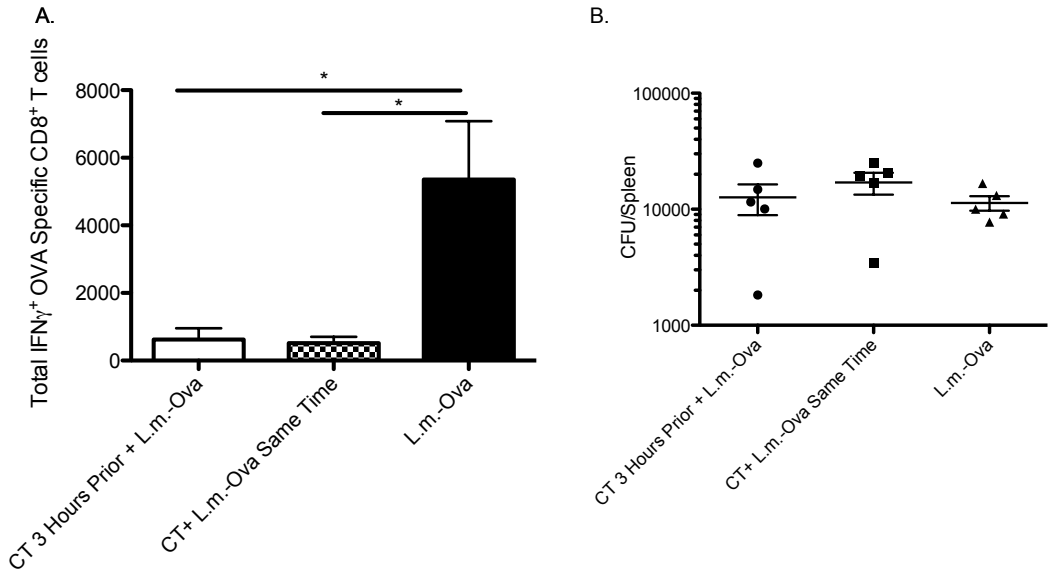

Figure 2-7 *C. trachomatis* infection reduces the *Listeria* specific CD8$^+$ T cell response in the dLNs during co-infection of the genital tract. Mice were transcervically infected with 10^6 IFU of *C. trachomatis* three hours prior to transcervical infection with 10^5 CFU of *L.m.*-Ova (left bar), or simultaneously infected (middle bar) or infected with 10^5 CFU of *L.m.*-Ova alone (right bar). A. Five days post infection the number of OVA specific IFNγ producing CD8$^+$ T cells were measured by ELISPOT. B. CFUs were measured in the spleen. *$p<0.05$ was determined by Mann-Whitney Test.

Additionally, I measured the number of activated CD8$^+$ T cells in the uterus by staining for CD44, a surface marker that is upregulated on activated T cells, and analyzed cells by flow cytometry. Mice that were infected with *C. trachomatis* three hours prior to *L.m.*-Ova infection had over a 2-fold decrease in the number of activated CD8$^+$ T cells present in the uterus compared to mice infected with *L.m.*-Ova alone. However, there were equivalent levels of activated CD4$^+$ T cells (**Figure 2-8**). Mice that were simultaneously coinfected with *C. trachomatis* and *L.m.*-Ova had similar levels of activated CD8$^+$ T cells and CD4$^+$ T cells. These data provide compelling evidence that

infection with *C. trachomatis* results in the general suppression of the $CD8^+$ T cell response in the genital tract.

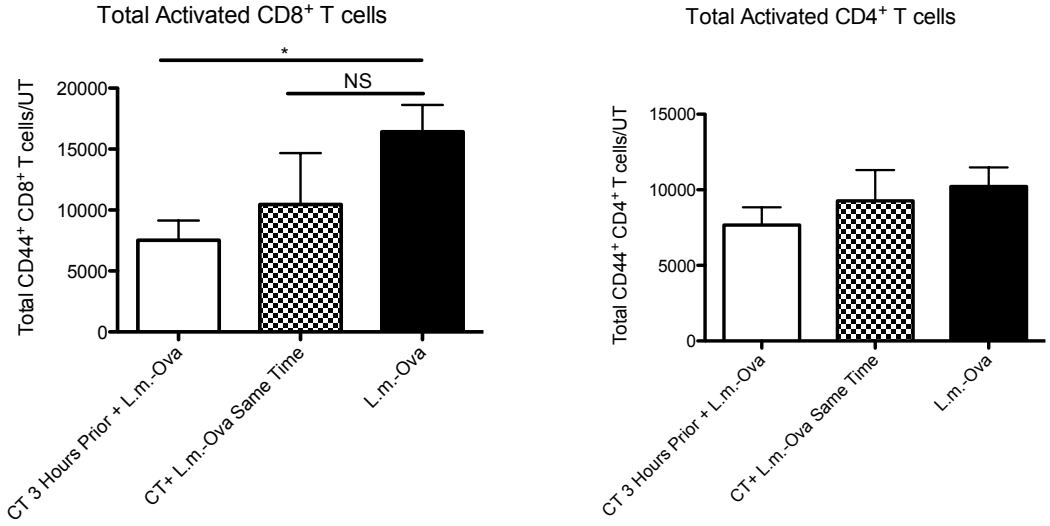

Figure 2-8 Infection with *C. trachomatis* impairs the $CD8^+$ T cells response to *L. monocytogenes.* Mice were transcervically infected with 10^6 IFU of *C. trachomatis* three hours prior to transcervical infection with 10^5 CFU of *L.m.-Ova* (left bar), or simultaneously infected (middle bar) or infected with 10^5 CFU of *L.m.-Ova* alone (right bar). Five days post infection the number of $CD44^+$ activated $CD8^+$ T cells (left graph) and $CD44^+$ activated $CD4^+$ T cells (right graph) were measured in the genital tract. *$p<0.05$ was determined by Mann-Whitney Test.

Characterizing the $CD8^+$ T cell response to *Listeria monocytogenes* infection of the genital tract

There are organs within the host that are considered immune-privileged because inflammatory responses in these organs can have especially negative consequences on fitness. Thus, in organs such as the brain and eye there are organ-driven mechanisms to restrain inflammatory responses (15, 16). It is possible that the upper genital tract, which

includes the uterus, may also be an immune-privileged site because excessive inflammation is likely to damage the reproductive tract and have negative impacts on fertility. In fact previous evidence suggested that TNFα producing $CD8^+$ T cells stimulated during *Chlamydia muridarum* infection contributes to inflammation and pathology of the uterus (17). However, the coinfection experiments presented above suggested that *C. trachomatis* specifically suppressed the $CD8^+$ T cell response. Therefore I wanted to test if the $CD8^+$ T cell response observed during transcervical *C. trachomatis* infection represented a general mechanism of the host to limit the $CD8^+$ T cell response in the genital tract. To test this idea, I examined the $CD8^+$ T cell response to *L. monocytogenes,* an organism known to elicit a protective $CD8^+$ T cell response, which can also infect the genital tract (18). In order to compare the $CD8^+$ T cell responses between *L. monocytogenes* and *C. trachomatis* I engineered a *L. monocytogenes* strain expressing the full length *CrpA* gene (*L.m.*-CrpA). I transcervically infected mice with 10^5 CFU of *L.m.*-CrpA and at multiple time points following primary infection, during memory and after secondary infection, I measured the number of CrpA specific $CD8^+$ T cells in the dLNs by flow cytometry. Similar to what was observed with the $CD8^+$ T cell response against *C. trachomatis, L.m.*-CrpA elicited a strong primary $CD8^+$ T cell response in the dLNs. By day seven post infection the $CD8^+$ T cell population had expanded over 3000 fold, and by day fourteen this population had returned to numbers similar to uninfected mice (**Figure 2-9).** After secondary challenge with *L.m.*-CrpA , the $CD8^+$ T cell population had an extremely blunted response compared to the primary infection, with an approximate six fold

decrease in the number of CrpA specific $CD8^+$ T cells five days following secondary infection compared to primary infection (**Figure 2-9).**

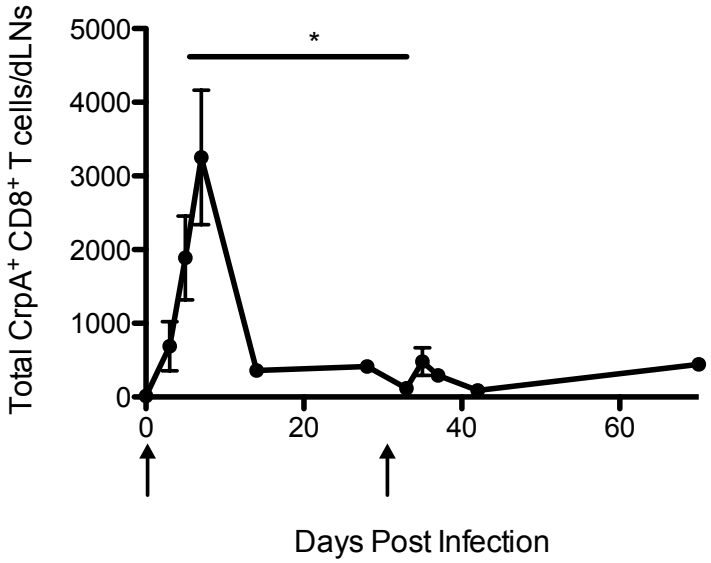

Figure 2-9: CrpA specific $CD8^+$ T cell response in the dLNs during *L.m.*-CrpA infection of the genital tract. Mice were transcervically infected with 10^5 CFU of *L.m.*-CrpA on day 0 (marked by 1st arrow). At time points marked, dLNs were harvested and the number of tetramer positive CrpA specific $CD8^+$ T cells were measured by flow cytometry. Mice where rechallenged with 10^5 CFU of *L.m.*-CrpA at the second arrow. Each time point is the mean of five mice per group, and error bars represent SEM. Statistical significance of $*p<0.05$ is indicated for day 5 primary and day 5 secondary infections by Mann-Whitney Test.

I next measured the $CD8^+$ T cell response during *L.m.*-CrpA infection in the genital tract by flow cytometry. By day seven post-primary infection the CrpA specific $CD8^+$ T cell population had expanded 5000 fold (**Figure 2-10).** The population quickly contracted and formed a memory population by day twenty-five that was about 100-fold higher than uninfected mice. Mice were rechallenged with *L.m.*-CrpA and the CrpA

specific $CD8^+$ T cell population quickly increased in the genital tract. By day five post-secondary infection, the CrpA specific $CD8^+$ T cell population was 4000 fold higher than day zero, to a total number significantly higher than day five post-primary infection (**Figure 2-10**). The secondary response was equally high on days five and seven post secondary infection, but contracted by day fourteen. The number of CrpA specific $CD8^+$ T cells measured on day seven post-primary and day seven post-secondary infections were not significantly different. These data show that the $CD8^+$ T cell secondary response to *L.m.*-CrpA infection of the genital tract occurs more quickly compared to the primary response. All together these data suggest that the impaired $CD8^+$ T cell response observed in the genital tract upon *C. trachomatis* infection may be specific to *C. trachomatis.*

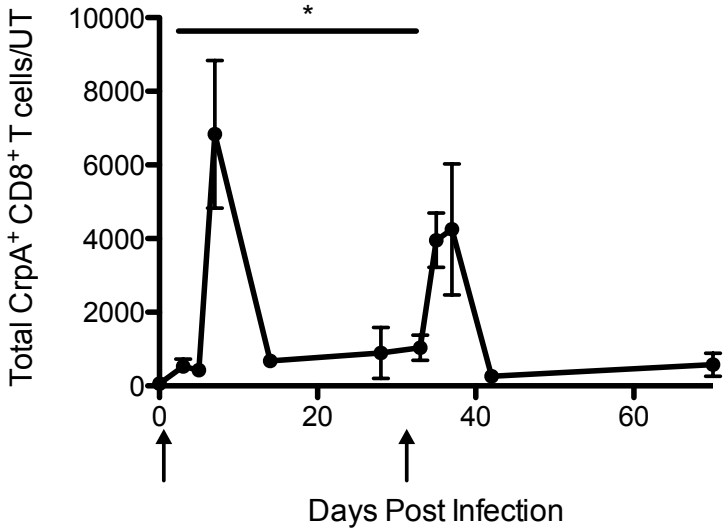

Figure 2-10: CrpA specific $CD8^+$ T cell response in the genital tract during transcervical *L.m.*-CrpA infection. Mice were transcervically infected with 10^5 CFU of *L.m.*-CrpA on day 0 (marked by 1st arrow). At time points marked, genital tracts were harvested and the number of tetramer positive CrpA specific

(Figure 2-10 Continued)

$CD8^+$ T cells were measured by flow cytometry. Mice where rechallenged with 10^5 CFU of *L.m.*-CrpA at the second arrow. Each time point is the mean of five mice per group, and error bars represent SEM. Statistical significance of *$p<0.05$ is indicated for day 5 primary and day 5 secondary infections by Mann-Whitney Test.

The $CD8^+$ T cell response in the dLNs was very similar between *C. trachomatis* and *L. monocytogenes* infection. However, the secondary $CD8^+$ T cell response in the genital tract differed between the two pathogens. Compared to *C. trachomatis, L.m.*-CrpA infection resulted in a larger memory population and elicited a faster and numerically higher secondary response. I hypothesized that these differences in secondary responses between *C. trachomatis* and *L. monocytogenes* infections may be in the phenotype of the $CD8^+$ T cells that are present in the genital tract during infection. To test this hypothesis, I transcervically infected mice with 10^6 *C. trachomatis*, 10^5 *L. monocytogenes,* or coinfected with both *C. trachomatis* and *L. monocytogenes.* Six days post infection, I analyzed the $CD8^+$ T cell responses by flow cytometry. $CD8^+$ T cells quickly develop memory phenotypes and can be broadly separated into two subsets based on the expression of CD127 (the IL-7 receptor) and CD62L (a lymph node homing marker). $CD8^+$ T cells that express high levels of CD127 and CD62L are known as central memory T cells ($T_{cm/n}$) and typically home to lymphoid organs, however, this population of cells may also contain naïve cells (T_n). $CD8^+$ T cells that express high levels of CD127 but low levels of CD62L are known as effector memory T cells (T_{em}) and migrate through peripheral tissues. Total numbers of $T_{cm/n}$ and T_{em} were measured in the genital tracts of infected mice and compared. Mice infected with *C. trachomatis* had a $T_{em}/T_{cm/n}$ ratio that was no different from uninfected mice. However, mice infected

with *L. monocytogenes* had a significantly higher ratio of T_{em} cells to $T_{cm/n}$ cells compared to mice infected with *C. trachomatis.* Simultaneous co-infection with *C. trachomatis* and *L. monocytogenes* led to a T_{em} to $T_{cm/n}$ ratio similar to *L. monocytogenes* infection alone (**Figure 2-11)**. Together these results indicate that *C. trachomatis* and *L. monocytogenes* infections produce phenotypically distinct $CD8^+$ T cell populations.

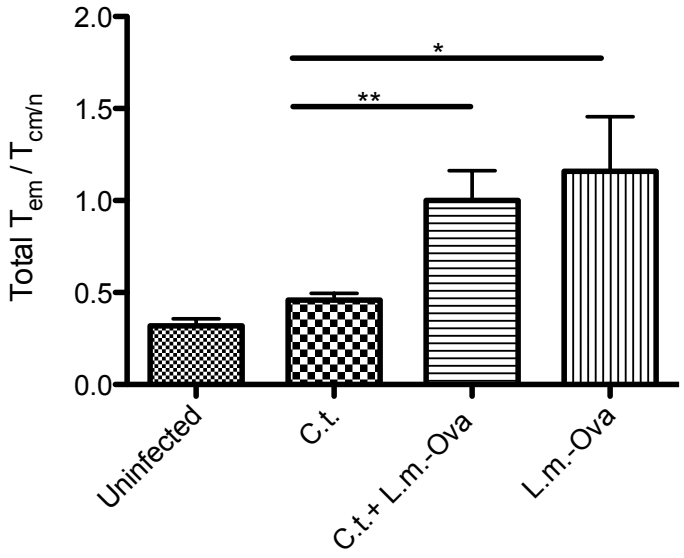

Figure 2-11 Transcervical infection with *L.m*-Ova produces a higher T_{em} to $T_{cm/n}$ ratio in the genital tract. Mice were either uninfected, transcervically infected with 10^6 IFU of *C. trachomatis,* simultaneously infected with 10^6 IFU of *C. trachomatis* and 10^5 CFU of *L.m*-Ova*,* or infected with 10^5 *L.m*-Ova alone. Six days post primary infection the total numbers of T_{em} ($CD127^+$, CD62L-) and $T_{cm/n}$ ($CD127^+$, $CD62L^+$) in the genital tracts were measured by flow cytometry. Bars show the mean ratio of total T_{em} over $T_{cm/n}$ of five mice per group, and error bars represent SEM. Statistical significance is indicated by *$p<0.05$ and **$p<0.01$, determined by Mann-Whitney Test.

Previous experiments demonstrated that infection with *C. trachomatis* prior to infection with *L.m.*-Ova resulted in fewer activated $CD8^+$ T cells in the genital tract (**Figure 2-8**). In order to understand if this reduction in the number of activated $CD8^+$ T cells correlated with differences in $CD8^+$ T cell phenotype, I transcervically infected mice with *C. trachomatis* three hours prior to *L.m.*Ova infection or simultaneously infected mice with both *C. trachomatis* and *L.m.*Ova . Five days post infection I measured the ratio of T_{em} to $T_{cm/n}$ in the genital tracts **(Figure 2-12).** Mice that received *C. trachomatis* three hours prior to *L.m.*Ova had a significant decrease in the ratio of T_{em} to $T_{cm/n}$ cells. Mice that were simultaneously infected with *C. trachomatis* and *L.m.*-Ova exhibited a similar T_{em} to $T_{cm/n}$ ratio compared to mice that received *L.m.*-Ova. All together these data suggest that prior infection with *C. trachomatis* alters the overall $CD8^+$ T cell response in the genital tract. Interestingly it is only mice that were infected with *C. trachomatis* three hours prior to *L.m.*-Ova that exhibited both a decrease in the number of activated $CD8^+$ T cells in the genital tract and a decrease in the T_{em} to $T_{cm/n}$ ratio, suggesting that activation status and $T_{em}/T_{cm/n}$ ratio could be connected.

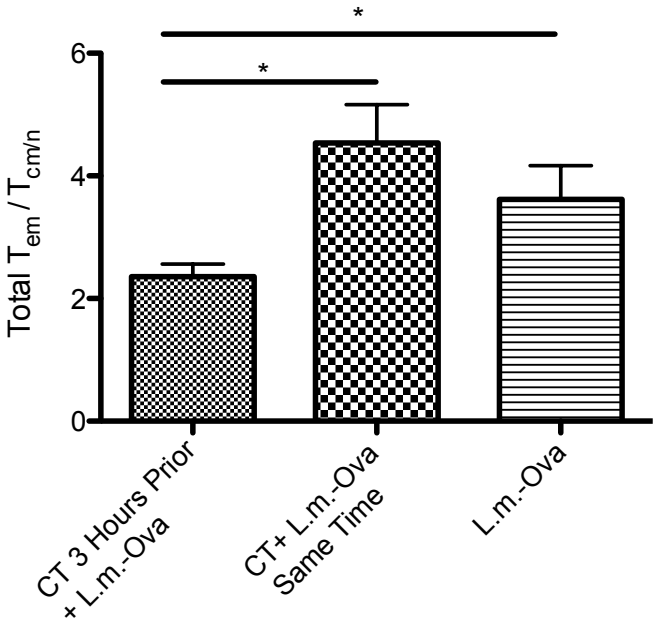

Figure 2-12 Prior infection with *C. trachomatis* alters the ratio of T_{em} to $T_{cm/n}$ during *L.m.*-Ova infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis* 3 hours prior to infection with 10^5 CFU of *L.m*-Ova, simultaneously infected with 10^6 IFU of *C. trachomatis* and 10^5 CFU of *L.m*-Ova, or infected with 10^5 *L.m*-Ova. Five days post primary infection the total numbers of T_{em} ($CD127^+$, CD62L-) and $T_{cm/n}$ ($CD127^+$, $CD62L^+$) in the genital tracts were measured by flow cytometry. Bars show the mean ratio of T_{em} over $T_{cm/n}$ of five mice per group, and error bars represent SEM. *$p<0.05$ was determined by Mann-Whitney Test.

Finally I wanted to test if the $CD8^+$ T cell response elicited by *L. monocytogenes* would be protective against rechallenge with *C. trachomatis.* I transcervically infected mice with *C. trachomatis, L.m*-CrpA, or *L.m.*-Ova. Four weeks after primary infection, I rechallenged mice with *C. trachomatis* and measured *C. trachomatis* burden in the genital tract five days later. As expected, mice that were previously infected with *C. trachomatis* showed significantly reduced bacterial levels compared to unimmunized mice (**Figure 2-13**). Interestingly, mice that received *L.m.*-CrpA or *L.m.*-Ova showed bacterial levels similar to mice that were previously infected with *C. trachomatis*. These data indicate

that infection with *L. monocytogenes* does protect against *C. trachomatis,* however it may not be dependent on the $CD8^+$ T cell response.

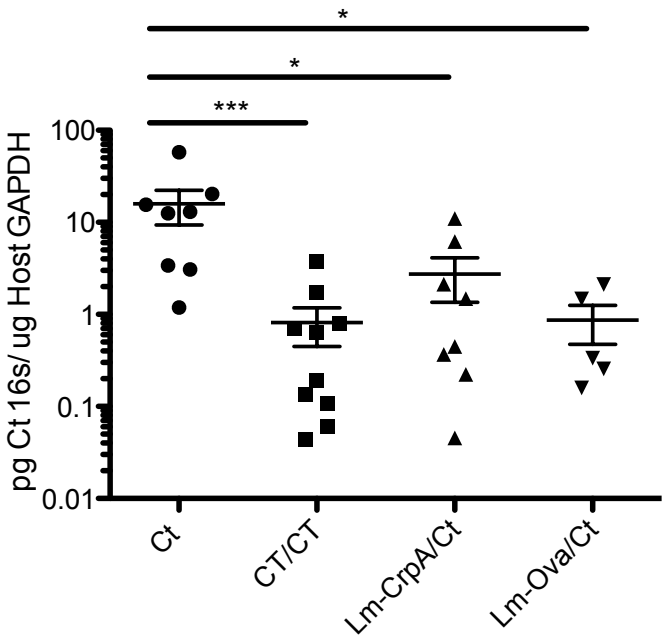

Figure 2-13 Immunization with *L. monocytogenes* protects mice against *C. trachomatis.* Mice were transcervically infected with 10^6 IFU of C. trachomatis, 10^5 CFU *L.m.*-CrpA, 10^5 CFU *L.m.*-Ova, or left uninfected. Four weeks after primary infections, mice were challenged with 10^6 IFU of *C. trachomatis;* five days later bacterial levels were measured by qPCR and normalized to host GAPDH levels. Shown is the compilation of 2 separate experiments, and error bars represent SEM. *$p<0.05$ and ***$p<0.0005$ were determined by Mann-Whitney Test.

Discussion

The hallmark of immune memory is the ability of the adaptive immune system to respond more quickly and vigorously to antigen to which it has been previously exposed. For *C. trachomatis,* there is ample evidence showing that patients can reacquire *C. trachomatis* infections, thus indicating a lack, or functional impairment, of immune memory to *C. trachomatis.* Systemic studies in mice showed that the $CD8^+$ T cell secondary response is blunted compared to the primary response. The studies in this chapter provide the first evidence indicating that the mucosal $CD8^+$ T cell response to *C. trachomatis* is also impaired.

Studies with acute viral pathogens such as Lymphocytic Choriomeningitis Virus (LCMV) and Influenza have provided the model for $CD8^+$ T cell responses. In these studies, primary infection with the viral pathogen produces a substantial $CD8^+$ T cell response. The virus is cleared, a stable $CD8^+$ memory population develops, and upon secondary challenge these memory cells are able to expand to greater numbers compared to primary infection thus clearing the virus before disease symptoms are apparent (9, 19). The data in this chapter clearly demonstrates that the secondary $CD8^+$ T cell response to *C. trachomatis* is blunted compared to the primary response and therefore does not mimic the model T cell response for these acute viral infections. In fact the $CD8^+$ T cell response to *C. trachomatis* infection does resemble that of chronic infections. For example, the $CD8^+$ T cell response to Hepatitis C Virus (HCV) is also described as dysfunctional. During HCV infection, antigen-specific $CD8^+$ T cells produce low levels of IFNγ. Although HCV specific $CD8^+$ T cells are present in infected patients, the levels

of $CD8^+$ T cells do not correlate with low viral titers, suggesting that $CD8^+$ T cells are unable to control HCV infection (20).

C. trachomatis is not the only sexually transmitted infection that results in impaired $CD8^+$ T cell responses that do not contribute to controlling pathogen burden. The Human Immunodeficiency Virus (HIV) is a genital pathogen that results in $CD8^+$ T cells that are unable to control viral replication (21). Human Papilloma Virus (HPV) also elicits $CD8^+$ T cells that have impaired cytokine production (22). However, the data from HPV and HIV studies were taken from human studies, in which these pathogens produce chronic disease. There are very few studies examining the $CD8^+$ T cell responses to *C. trachomatis* in humans. Though, there is evidence that $CD8^+$ T cells from *C. trachomatis* infected patients are low in lytic cytokine production (23).

The impaired $CD8^+$ T cell response to *C. trachomatis* infection is similar to that of chronic pathogens, however in mice *C. trachomatis* produces an acute infection. Thus it was unclear if the observed $CD8^+$ T cell response was representative of a *C. trachomatis* specific effect or a typical $CD8^+$ T cell response to a genital infection. Therefore *L. monocytogenes* was chosen in order to do a comparative analysis. Infection with *L. monocytogenes* produces a well-described protective $CD8^+$ T cell response (24, 25). It was hypothesized that if the genital tract was an immune-suppressive environment than the $CD8^+$ T cell response to *L. monocytogenes* would be similar to that of *C. trachomatis.* Transcervical infection with *L. monocytogenes* produced a $CD8^+$ T cell response in the dLNs that was identical to that of *C. trachomatis.* Interestingly, the $CD8^+$ T cell response in the genital tract differed between the two organisms. Most striking was the enhanced $CD8^+$ T cell response in the genital tract during secondary *L.*

monocytogenes infection compared to primary infection, this is in comparison to the extremely blunted $CD8^+$ T cell response to secondary *C. trachomatis* infection. These data provide several interesting hypotheses. The fact that the secondary $CD8^+$ T cell responses in the dLNs to both *C. trachomatis* and *L. monocytogenes* were blunted suggests that the secondary expansion and T cell recruitment could occur outside of the dLNs, possibly in the genital tract. This is supported by the fact that there is very little expansion or retention of the $CD8^+$ T cell population in the dLNs during secondary *L. monocytogenes* infection, yet there is a robust secondary population present in the genital tract. Furthermore, during *L. monocytogenes* infection there is a larger memory $CD8^+$ T cell population that is maintained in the genital tract compared to *C. trachomatis* infection, even though both infections produced similar numbers of CrpA specific $CD8^+$ T cells during primary infections. The increase in the memory $CD8^+$ T cell population during *L. monocytogenes* infection may account for the larger secondary response upon rechallenge. However, future studies should test this hypothesis by directly measuring the expansion of *L. monocytogenes* specific memory $CD8^+$ T cells transferred into a naïve host. All together these data support that the $CD8^+$ T cell response to *C. trachomatis* is specific to the pathogen and does not represent the response generated by another pathogen.

The ultimate question that remains is why is there a larger memory $CD8^+$ T cell population and larger secondary $CD8^+$ T cell response during *L. monocytogenes* infection compared to *C. trachomatis*? Understanding the answer to this question may allow researchers to manipulate a protective $CD8^+$ T cell response to *C. trachomatis.* The studies in this chapter begin to describe the differences between *L. monocytogenes* and *C.*

trachomatis that could contribute to the different recall capacities. During *L. monocytogenes* infection, the $CD8^+$ T cells are skewed to a more T_{em} phenotype, whereas *C. trachomatis* infection produces a $CD8^+$ T cell population that is skewed toward a $T_{cm/n}$ phenotype. The higher T_{em} ratio in *L. monocytogenes* infection may account for the larger memory population present in the genital tract. By expressing lower levels of CD62L, T_{em} cells are more likely to reside in, or migrate through, the peripheral tissue. Previous studies have demonstrated that in mucosal tissues T_{em} cells require less stimulation and are more quickly able to differentiate into cytokine producing effector cells when compared to $T_{cm/n}$ cells (26, 27). $T_{cm/n}$ on the other hand express high levels of CD62L, which allow these cells to circulate systemically, but therefore result in these cells being physically separated from the mucosal site of infection (26). In fact, previous studies have demonstrated that HPV may evade the immune response by skewing the $CD8^+$ T cell response towards the T_{cm} phenotype, thus excluding them from the genital mucosa (22). The data presented in this chapter suggest that *C. trachomatis* infection skews the $CD8^+$ T cells to a $T_{cm/n}$ phenotype, and this may contribute to the impaired $CD8^+$ T cell recall response. This may also explain why mice that are infected with *C. trachomatis* prior to *L. monocytogenes* infection produce fewer activated $CD8^+$ T cells in the genital tract, however when mice are simultaneously coinfected the $CD8^+$ T cell response is skewed toward a T_{em} phenotype and there are more activated $CD8^+$ T cells in the genital tract. Although correlative, these data suggest that the levels of activated $CD8^+$ T cells may be related to the populations of T_{em} to $T_{cm/n}$ present in the genital tract. Future experiments should use activation markers to distinguish naïve T cells from central memory $CD8^+$ T cells in the genital tract of mice infected with *C. trachomatis*

and/or *L. monocytogenes*. It is possible that the $T_{cm/n}$ population in *C. trachomatis* infected mice is primarily naïve cells, which would indicate that primary infection fails to properly prime the $CD8^+$ T cell response. Finally, the T_{em} skewed $CD8^+$ T cell response elicited by *L. monocytogenes* may partially explain why infection with this organism, lacking a known *C. trachomatis* antigen, still confers protection against *C. trachomatis.* Previous studies have demonstrated that *L. monocytogenes* specific memory $CD8^+$ T cells expressing low levels of CD62L mediate rapid, non-antigen specific IFNγ responses (27, 28). This suggests that the *L. monocytogenes* specific memory $CD8^+$ T cells may non-specifically secrete IFNγ that leads to protection against *C. trachomatis,* although future experiments will test this hypothesis. Furthermore, it will be interesting for future studies to discern if T_{cm} and T_{em} cells generated by *L. monocytogenes* infection provide different levels of protection against *C. trachomatis* and exhibit differences in bystander activity. It will be important to understand if bystander activity from memory $CD8^+$ T cells contributes to the mucosal immune response and mediates early control of pathogen replication in the genital tract.

The evidence presented here leads to the hypothesis that *C. trachomatis* infection impairs the recall capacity of $CD8^+$ T cells, yet how that occurs is not clearly understood. To fully investigate this question it will be necessary to distinguish the kinetics of the secondary response. The recall response following *C. trachomatis* secondary infection could be due to a lack of expansion of the memory cells, an impairment of naïve cells that also contribute to the secondary response, a migration defect or increased cell death of memory cells. In order to discern between these possibilities future studies should examine $CD8^+$ T cell expansion in the genital tract by directly measuring dilution of the

molecule Carboxyfluorescein succinimidyl ester (CFSE) of memory $CD8^+$ T cells transferred into naïve mice. Furthermore, coupling this experiment with apoptosis markers will allow researchers to investigate if there is increased death of memory $CD8^+$ T cells. Another possible explanation for the lack of recall response to *C. trachomatis* infection is that the secondary expansion is happening outside the genital tract. The experiments here have excluded the genital tract and dLNs, but it is possible that secondary expansion occurs in another tissue such as the spleen. Investigating the presence of *C. trachomatis* specific $CD8^+$ T cells in other tissues following secondary infection will provide insight into the possibility that memory $CD8^+$ T cells are defective in their ability to migrate to the genital tract.

What these experiments, and those of others, have not yet demonstrated is the existence of resident memory $CD8^+$ T cells in the upper genital tract. In other mucosal surfaces, such as the skin and intestines, there are memory $CD8^+$ T cells that permanently reside in these tissues. During infection, resident memory $CD8^+$ T cells are poised to act quickly in order to control infection. Whether the T_{em} populations elicited by *C. trachomatis* and *L. monocytogenes* are actually resident memory populations is unclear. Future experiments can explore this possibility by comparing the ability of T_{em} and T_{cm} $CD8^+$ T cell populations isolated from the genital tracts of *C. trachomatis* or *L. monocytogenes* infected mice to migrate to the genital tract and provide protection in naïve mice. Alternatively, by using a parabiosis model it will be possible to distinguish what cellular populations from immune mice are able, or unable, to migrate to the naïve parabiosed partner. Ultimately the experiments in this chapter lay the foundation for

future studies that will enable researchers to understand the factors that determine a protective $CD8^+$ T cell response in the genital tract.

Methods

Mice

C57BL/6 mice were purchased from The Jackson Labs. IrgM1/3 deficient mice have been described previously (14). All animals were maintained and cared for within the Harvard Medical School Center for Animal Resources and Comparative Medicine (Boston, MA). All mice were treated with 2.5 mg medroxyprogesterone subcutaneously 7 days prior to infection to synchronize the murine estrous cycle. All experiments were approved by the Institutional Animal Care and Use Committee.

Growth, isolation, and detection of bacteria

C. trachomatis serovar L2 (434/Bu) was propagated within McCoy cell monolayers grown in Eagle's MEM (Invitrogen, Grand Island, NY) supplemented with 10% FCS, 1.5 g/l sodium bicarbonate, 0.1M nonessential amino acids, and 1 mM sodium pyruvate. Infected monolayers were disassociated from plates, using sterile glass beads, and were sonicated to disrupt the inclusion. Elementary bodies were purified by density gradient centrifugation, as described previously (29). Aliquots were stored at -80°C in medium containing 250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid (SPG) and were thawed immediately prior to use. To quantify the levels of *C. trachomatis* quantitative PCR with 16S primers specific for *Chlamydia* was performed as has been previously described (29). *Listeria monocytogenes* 10403S, and *L.m.*-Ova were provided by Darren Higgins. *L. monocytogenes* strains were grown in Brain Heart Infusion media (BHI) and passaged through mice. Prior to infection frozen aliquots of *L.*

monocytogenes were incubated in 10ml of BHI at 37°C for 1 hour with shaking. Based on the OD 600, dilutions of *L. monocytogenes* were made in SPG. To quantify levels of *L. monocytogenes* after infection, uterine tissue was homogenized in 2 ml of HBSS and 80µl aliquots were plated on BHI containing 100µg/ml of streptomycin.

Listeria monocytogenes-CrpA strain creation

The CrpA gene was amplified from *C. trachomatis* serovar L2 (434/Bu) by PCR using the following primers; Fwd: 5'-GGGCGGCCGAAATGAGCACTGTACCCGTTGT-3'; Rev: 5'-GGGCGGCCGTTTGGGTCTGATCCACCA-3'. The amplification added EagI restriction sites to both the 5' and 3' ends. The construct was then ligated into the pVEV vector, created from the pPL2 integration vector and generously provided by Darren Higgins (30, 31). The pVEV vector contains the ActA promoter and signaling sequences. This created a construct in which the CrpA sequence is fused between the ActA signal sequence and the OVA SIINFEKL sequence. The vector was then integrated into the *L. monocytogenes* chromosome by electroporation with the following settings: 1 KV, 400 ohms, 25 mFD. The resulting strain was maintained on BHI containing 7.5µg/ml of chloramphenicol and 100µg/ml of streptomycin. Secretion of CrpA-OVA fusion was confirmed by CrpA-tetramer staining and OVA-pentamer staining of splenocytes isolated from mice three days post intraveneous infection.

Flow cytometry

Tissues were mechanically disaggregated and immediately stained for surface markers. For ICCS, tissues were stimulated for 5 h with 50 ng/ml PMA (Alexis Biochemical) and 500 ng/ml ionomycin (Calbiochem) in the presence of brefeldin A (GolgiStop; BD Biosciences) for intracellular cytokine staining. Cells were preincubated with anti-FcRg (Bio X-Cell) before staining with αCrpA-APC, αCD4 Q-Dot, αCD8-Pe-Cy7, and αCD3-Pe (Biolegend). Cells were also incubated with αCD11b-PB, αCD11c-PB, αCD19-PB and αB220-PB (Biolegend) as a negative gate. For activation marker analysis, cell were incubated with αCD62L-ApcCy7 (Biolegend), αCD127-PerCP-Cy5.5 (BD Biosciences), and αCD44-FITC (Biolegend). For intracellular staining αIFN-g PE (BD Biosciences) was used. Cells were permeabilized with the Cytofix/Cytoperm Plus Kit according to the manufacturer's instructions (BD Biosciences). The absolute cell number in each sample was determined using AccuCheck Counting Beads (Invitrogen). Data were collected on an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star). APC-CrpA tetramer was provided by the National Institute of Health Tetramer Core Facility.

ELISPOT assays

ELISPOT filter plates were incubated overnight with 10μg/ml of anti-IFNγ capture antibody (Biolegend). Plates were blocked with RPMI media for 1 hour. EL4 cells were incubated for one hour with 10μM of CrpA or OVA peptide, the cells were then treated for one hour with 100μg/ml Mitomycin C. EL4 cells were washed several times and then plated at 10^5 cells per well. Lymphocytes were harvested from infected mice and plated in triplicate with $5x10^5$ and $2.5x10^5$ lymphocytes per well. Twenty-four

hours later, plates were washed and then incubated with 10μg/ml of biotin conjugated anti-IFNγ antibody overnight at 4°C. Plates were then incubated with Streptavidin-HRP for 1 hour at room temperature. Plates were developed by incubating with BCIP/NBT solution (25mg in 10ml of H_2O). Spots were counted using a dissecting microscope.

T cell depletion

For $CD8^+$ T cell depletion experiments, mice depleted prior to primary challenge were injected i.p. with 200μg of anti-CD8 (clone 2.43) or isotype control (clone LTF-2) from BioXCell. For primary challenge, mice were treated with antibody every day starting 3 days prior to primary challenge and then every other day after challenge. Mice were sacrificed 5 days after challenge. For secondary challenge, mice were treated every day for 3 days prior to secondary challenge and then every other day after secondary challenge.

Statistical analysis

The data represent the mean ± SEM and were calculated using GraphPad Prism version 4.0. *P* values were determined using the nonparametric Mann-Whitney *U* test. Significant differences between groups are indicated as follows: *$p<0.05$, **$p< 0.01$, and ***$p<0.005$.

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Chapter Three: Expression of the immuno-inhibitory ligand, PD-L1, is upregulated in the genital tract during *C. trachomatis* infection.

PD-L1 limits the mucosal CD8$^+$ T cell response to *Chlamydia trachomatis*

Sarah Fankhauser and Michael Starnbach

Portions of this chapter have been submitted as part of a manuscript to the Journal of Immunology. The project was conceived by S. Fankhauser and M. Starnbach. Experiments and data analysis were performed by S. Fankhauser, and all text and figures were produced by S. Fankhauser.

Introduction

In mice, *Chlamydia trachomatis* infection stimulates $CD8^+$ T cells that expand over 100 fold, and subsequently contract to form a memory population (1, 2). However, upon rechallenge with *C. trachomatis,* the secondary $CD8^+$ T cell response is significantly smaller in magnitude, with fewer cytokine producing $CD8^+$ T cells, compared to the primary $CD8^+$ T cell response (2). This type of impaired secondary $CD8^+$ T cell response is reminiscent of chronic infections with viral pathogens such as Human Immunodeficiency Virus (HIV) and Lymphocytic Choriomeningitis Virus Clone 13 (LCMV-Cl13). The memory $CD8^+$ T cells that develop after HIV and LCMV-Cl13 infections exhibit an exhausted phenotype defined by low cytokine production, low replicative potential, and increased expression of pro-apoptotic genes, all of which lead to extremely deficient secondary $CD8^+$ T cell responses that are unable to control pathogen replication (3-5).

A significant cause of defective $CD8^+$ T cell responses in chronic viral infections, such as HIV and LCMV-Cl13, is the engagement of immunoinhibitory pathways (6-9). The engagement of immunoinhibitory pathways can directly inhibit T cell signaling produced by the costimulatory molecules, or affect signaling pathways downstream that can lead to apoptosis, reduced cytokine production, and altered memory responses (10, 11). A well-described immunoinhibitory pathway is made up of the receptor PD-1 and its ligand PD-L1. PD-1 expression can be induced on T cells upon activation, and PD-L1 is expressed on a wide variety of cells including professional antigen presenting cells (pAPC), epithelial and endothelial cells (12-14). It is unclear if the PD-1/PD-L1 pathway

or any other known immunoinhibitory pathways are responsible for producing the impaired secondary $CD8^+$ T cell response during *C. trachomatis* infection. I hypothesized that the blunted $CD8^+$ recall response, and lack of $CD8^+$ T cell contribution to infection control, is due to the engagement of one or more immunoinhbitory pathways. In this chapter, I determine which, if any, immunoinhibitory ligands and receptors are highly expressed during *C. trachomatis* infection. Immunoinhibitory ligands or receptors that are highly expressed upon *C. trachomatis* infection are potential candidate pathways that may function to alter the $CD8^+$ T cell response to *C. trachomatis.*

Results

Identification of immunoinhibitory molecules upregulated during *C. trachomatis* infection

It is clear that *C. trachomatis* is able to stimulate a $CD8^+$ T cell response (**Chapter 2 Figures 2-1 and 2-2**). However, our data and that of others have shown that this $CD8^+$ T cell response is impaired during *C. trachomatis* infection. Different studies have demonstrated that the activation of immunoinhibitory pathways can directly inhibit the $CD8^+$ T cell response against different pathogens (4, 5). I hypothesized that an immunoinhibitory pathway is upregulated during *C. trachomatis* infection. I took a candidate approach and selected to study the expression of ligands and receptors that have been previously well described as having a role in restricting $CD8^+$ T cell responses. I transcervically infected mice with 10^6 *C. trachomatis* and examined the expression of selected immunoinhibitory receptors and ligands by quantitative real time reverse transcriptase-PCR (qRT-PCR) in whole uterine tissue at the peak of bacterial burden, five days post-infection (**Figure 3-1**). Several genes exhibited altered expression in infected mice compared to mock infected mice. The expression of 2B4, CD160 and Lag3 was significantly upregulated in infected mice, and the expression of PD-L2 and PD-1 was down-regulated in infected mice. Interestingly, the immunoinhibitory ligand PD-L1, which binds to PD-1, was expressed over 10 fold higher in the uteri of infected mice compared to mock infected mice (**Figure 3-1**). I chose to examine PD-L1 expression further, and this will be the focus for the remaining chapter.

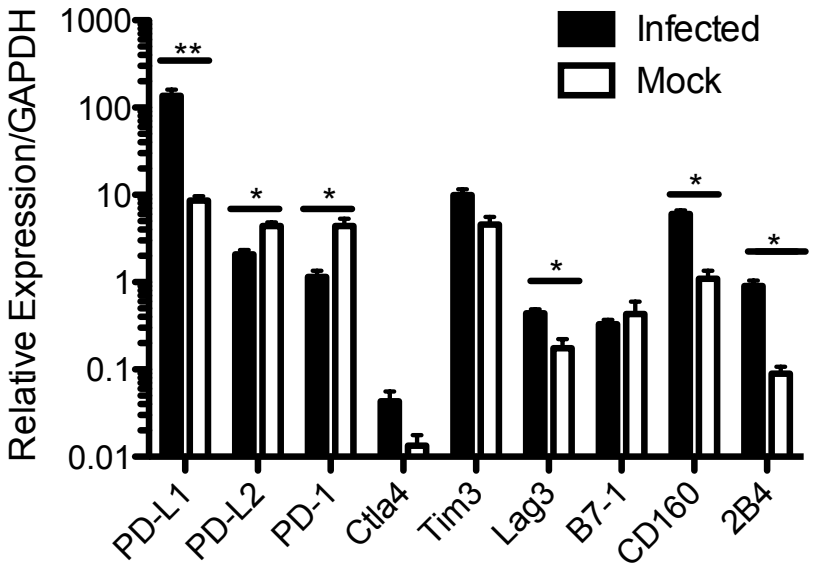

Figure 3-1 Inhibitory molecule expression during primary *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis* or mock infected. Five days post infection RNA was extracted from uterine tissue, qRT-PCR was performed using primers specific for the indicated genes and expression levels were normalized to host GAPDH expression levels. Bars represent the mean of four mice. Error bars indicate SEM. Statistical significance is indicated by *$p<0.05$ and **$p<0.01$ by Mann-Whitney Test.

PD-L1 upregulation is dependent on infection with live *C. trachomatis* infection

Previous work has suggested that PD-L1 expression limits the inflammatory response to *Chlamydia muridarum,* therefore PD-L1 expression was investigated further (15). I questioned whether PD-L1 expression was dependent on live *C. trachomatis* infection or just the result of a non-specific inflammatory response to bacterial antigen. I transcervically infected mice with either live or UV-inactivated *C.trachomatis* and assessed PD-L1 expression in the uterus by qRT-PCR five days after infection. PD-L1 expression in mice infected with UV-inactivated *C. trachomatis* was similar to mock-

infected mice, thus confirming that the increase in PD-L1 expression is dependent on live *C. trachomatis* infection (**Figure 3-2**).

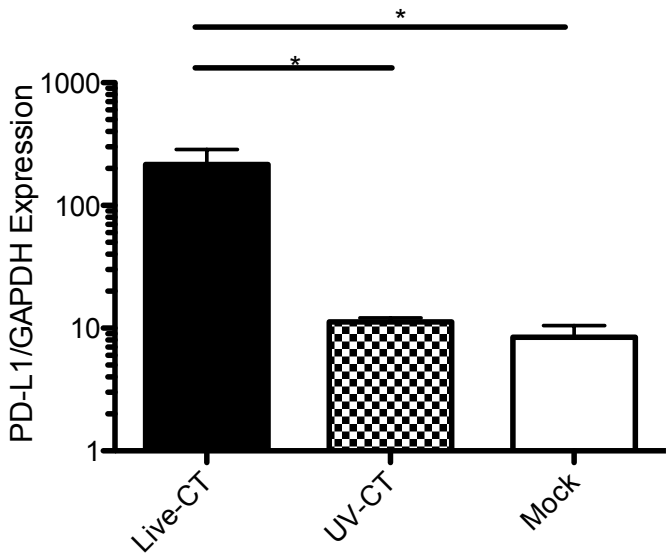

Figure 3-2 PD-L1 upregulation is dependent on live *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of live *C. trachomatis,* 10^6 IFU of UV-inactivated *C. trachomatis,* or mock infected. Five days post infection RNA was extracted from uterine tissue, qRT-PCR was performed using primers specific for PD-L1 and expression levels were normalized to host GAPDH levels. Bars represent the mean of five mice. Error bars indicate SEM. Statistical significance is indicated by *$p<0.05$ by Mann-Whitney Test.

To test if infection alone, independent of host immune responses, was sufficient to cause PD-L1 upregulation, I infected a cultured thymic epithelial cell line with live *C. trachomatis* or UV-inactivated *C. trachomatis*. Eighteen hours after infection I measured PD-L1 surface expression by flow cytometry and compared this to uninfected cells. Similar to the *in vivo* data, PD-L1 was highly expressed on cells infected with live *C. trachomatis*, but not on cells infected with UV-inactivated *C. trachomatis* or uninfected

cells (**Figure 3-3**). These data provide strong evidence that *C. trachomatis* infection directly upregulates PD-L1 expression.

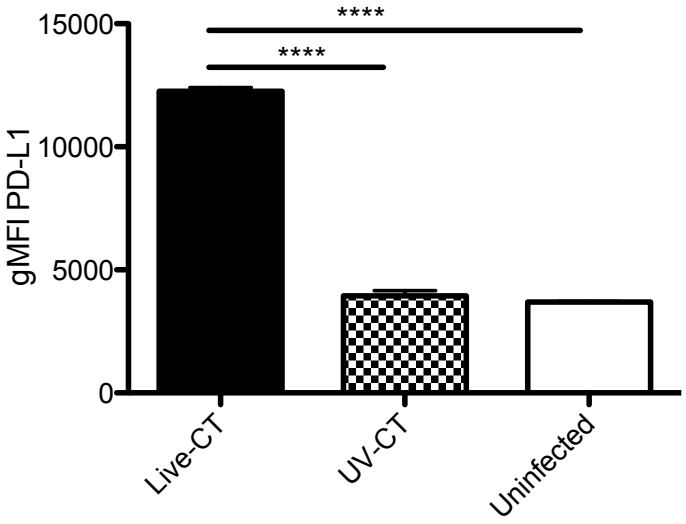

Figure 3-3 PD-L1 surface expression is upregulated on cultured epithelial cells 18 hours post *C. trachomatis* infection. Cultured 1308.1 cells were infected at an MOI of 1:1 with live or UV-inactivated *C. trachomatis.* 18 hours post infection PD-L1 surface expression, indicated as geometric mean fluorescent intensity (gMFI), was measured by flow cytometry. Bars represent the mean of three different wells, and error bars indicate SEM. Data are representative of two independent experiments. Statistical significance is indicated by ****$p<0.0001$ by a Two-Tailed Students T test.

PD-L1 is expressed on multiple cell types upon *C. trachomatis* infection

Next I wanted to determine which cell types were responsible for the observed PD-L1 upregulation during *C. trachomatis* infection. PD-L1 can be expressed on a variety of cell types such as dendritic cells, $CD4^+$ T cells, endothelial cells and epithelial cells. Different cell types lead to variation in PD-L1 signaling properties (12, 16, 17). I therefore wanted to examine the expression of PD-L1 on different cellular populations

within the uterus and draining lymph nodes (dLNs). I transcervically infected mice with *C. trachomatis*; at different time points after primary and secondary infection I harvested the uteri and dLNs and measured PD-L1 surface expression by flow cytometry. Five days after both primary and secondary infection PD-L1 surface expression increased five fold on uterine epithelial cells from infected mice compared to uninfected mice (**Figure 3-4**). PD-L1 expression on DCs, $CD4^+$ T cells and $CD8^+$ T cells in the uterus was comparable between infected and uninfected mice. PD-L1 surface expression was also significantly higher on DCs in the dLNs five days after primary and secondary infection (**Figure 3-5**). However, twenty-seven days after primary infection, during the memory phase, there was no difference in PD-L1 expression compared to uninfected mice **(Figures 3-4 and 3-5**). These data demonstrate that PD-L1 is highly expressed on uterine epithelial cells and dLN DCs during primary and secondary *C. trachomatis* infection, but this elevated expression is not maintained after infection is cleared.

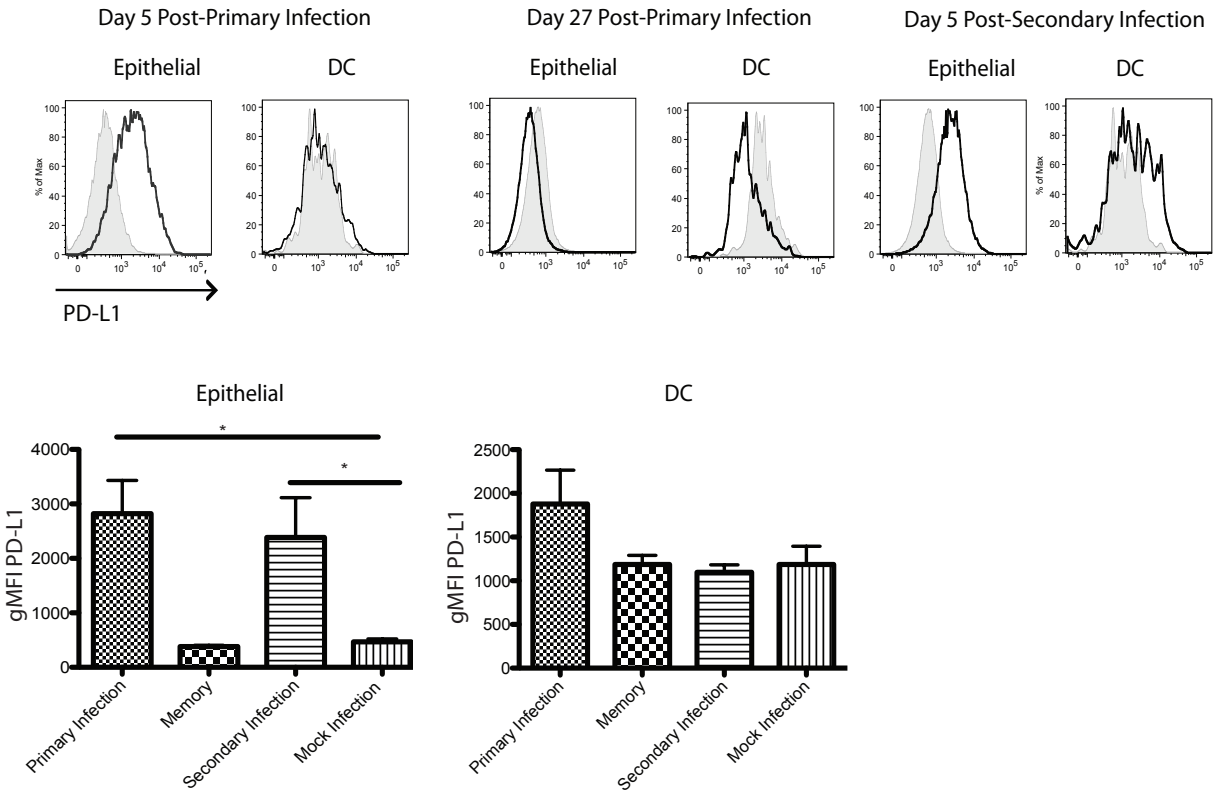

Figure 3-4 PD-L1 is highly expressed on uterine epithelial cells upon transcervical *C. trachomatis* infection. Mice were infected with 10^6 IFU of *C. trachomatis* and at indicated time points post infection, uteri were examined for PD-L1 expression by flow cytometry. Epithelial cells were gated on live $CD326^+$ populations, DCs were gated on live, $CD45^+$, $CD4^-$, $CD11^+$ lymphocytes. Shown are representative histograms of PD-L1 expression. Black line represents infected mice, gray shaded histrogram represents mock infected mice. Bar graphs show the average gMFI of PD-L1 expression on uterine epithelial cells (left) and uterine DCs (right) from five mice per time point. Error bars represent SEM. Data are representative of two independent experiments. Statistical significance is indicated by *$p<0.05$ by Mann-Whitney Test.

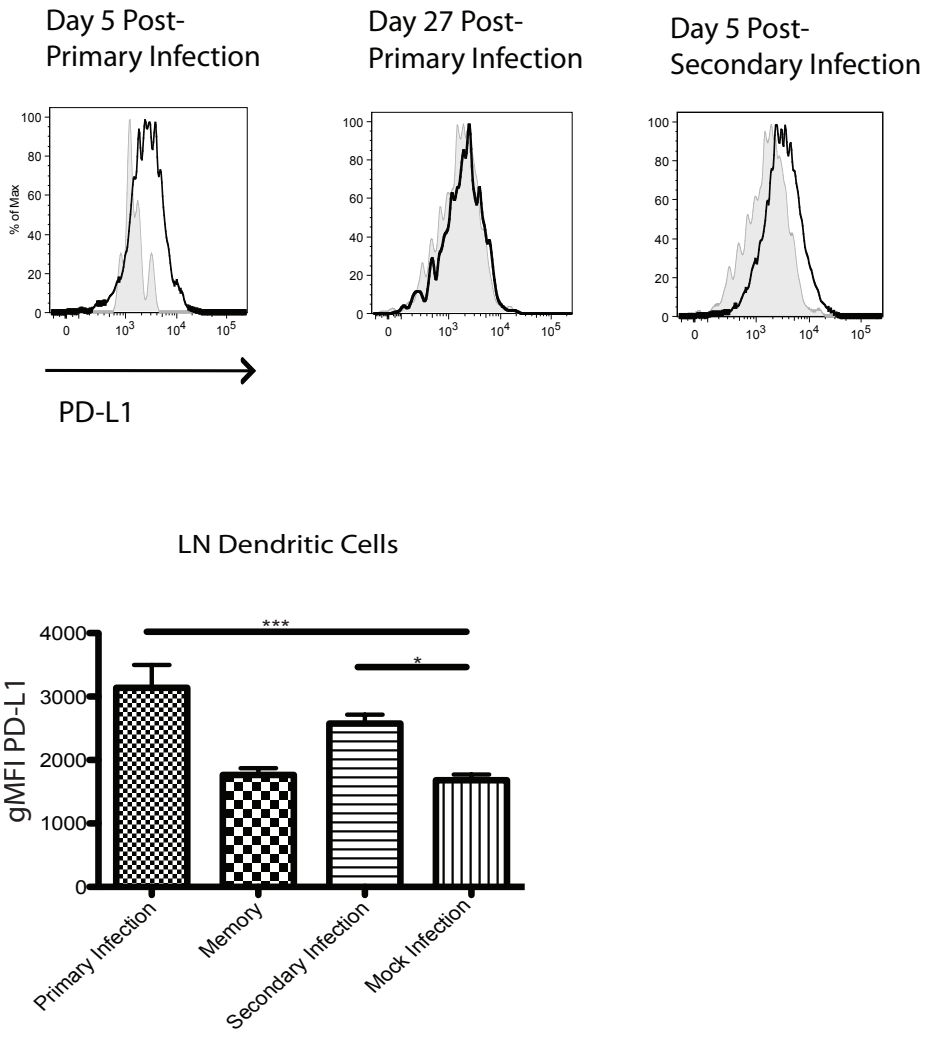

Figure 3-5 PD-L1 is highly expressed on dendritic cells of the dLNs upon transcervical *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis* and at indicated time points post infection, dLNs were examined for PD-L1 expression by flow cytometry. Dendritic cells were gated on live CD45.2$^+$, CD11c$^+$, CD4$^-$ lymphocytes. Shown are representative histograms of PD-L1 expression. Black line represents infected mice, gray shaded histrogram represents mock mice. Bar graphs show the average gMFI of PD-L1 expression on CD11c$^+$ DCs from five mice per time point. Error bars represent SEM. Data are representative of two independent experiments. Statistical significance is indicated by *p<0.05 by Mann-Whitney Test.

Priming of the $CD8^+$ T cell response occurs very early during infection. Although the kinetics of priming are not known for *C. trachomatis,* for the bacterial pathogen *Listeria monocytogenes* $CD8^+$ T cells are primed by pAPCs between 24 and 48 hours of infection (18). I hypothesized that PD-L1 expression on dLN DCs could alter priming of the $CD8^+$ T cells during *C. trachomatis* infection. If this were true then PD-L1 upregulation must occur very quickly upon infection. To test this, PD-L1 surface expression was measured 20 hours after primary infection. At this early timepoint, PD-L1 surface expression was upregulated on dLN dendritic cells and uterine epithelial cells when compared to uninfected mice (**Figure 3-6**). The early expression of PD-L1 on dLN DCs suggests that this molecule could impact the priming and development of the $CD8^+$ T cell response.

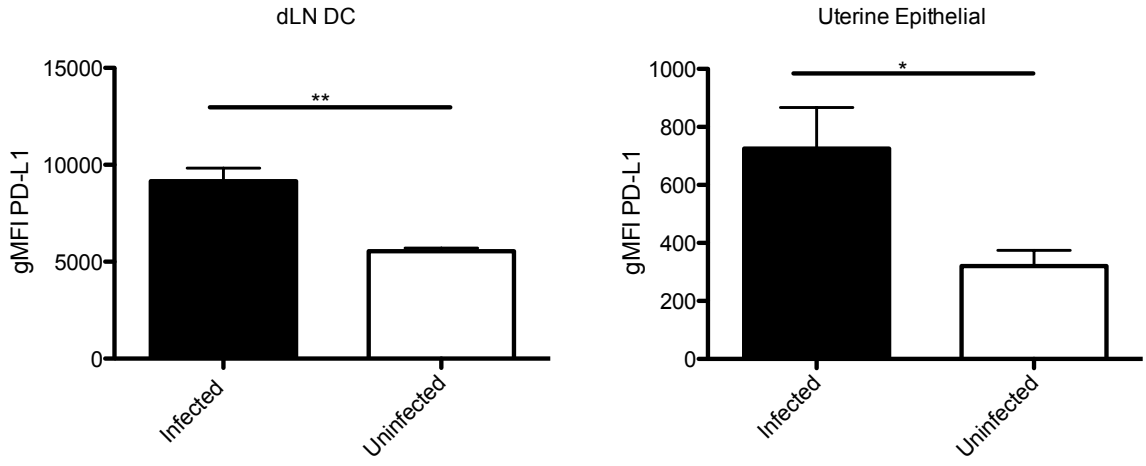

Figure 3-6 PD-L1 surface expression is upregulated 20 hours post transcervical *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis.* Twenty hours post infection, dLN DCs (left panel) and uterine epithelial cells (right panel) were examined for PD-L1 expression by flow cytometry. Bar graphs show the average gMFI of PD-L1 from five mice per group. Error bars represent SEM. Statistical significance is indicated by *$p<0.05$ and **$p<0.01$ by Mann-Whitney Test.

Previous studies have reported that PD-L1 expression depends on IFNγ signaling (19, 20). There are two main pieces of evidence to suggest that PD-L1 upregulation is independent of IFNγ during *C. trachomatis* infection. First, PD-L1 surface expression is upregulated during *C. trachomatis* infection of tissue culture cells, which produce very low levels of IFNγ (data not shown). Second, there is no increase in PD-L1 surface expression during infection with UV-inactivated *C. trachomatis,* which still produces a potent inflammatory response. To test this hypothesis, I transcervically infected WT and IFNγ-deficient mice with *C. trachomatis.* Five days post-infection, PD-L1 surface expression was measured by flow cytometry. Interestingly, there was a significant upregulation of PD-L1 expression on dLN DCs in infected IFNγ-deficient mice, compared to mock infected IFNγ-deficient mice (**Figure 3-7**). Furthermore, the level of PD-L1 surface expression on uterine epithelial cells measured in infected IFNγ-deficient mice was similar to that of infected WT mice, and significantly higher than mock infected mice (**Figure 3-7**). Together these data indicate that there is an IFNγ independent upregulation of PD-L1 expression during *C. trachomatis* infection.

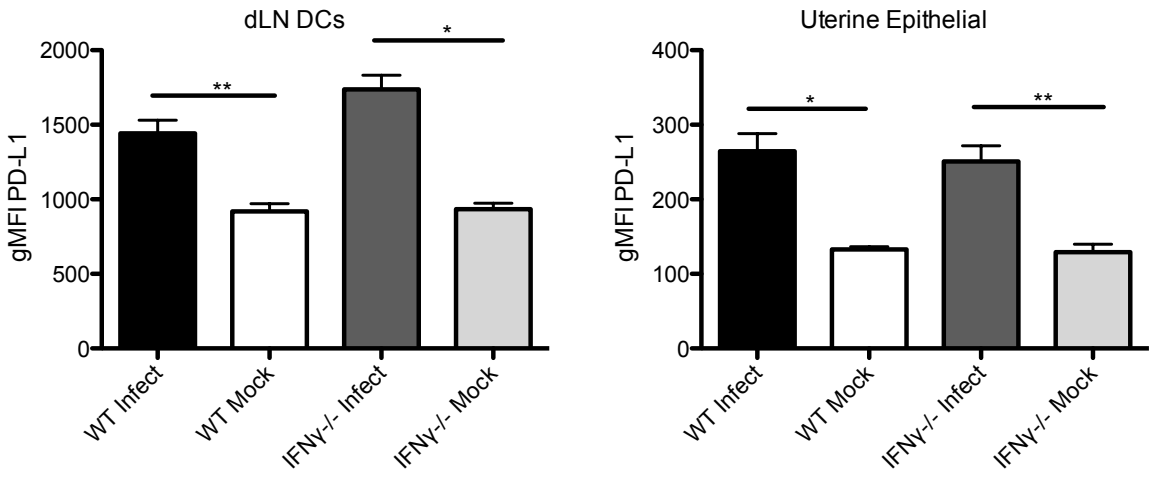

Figure 3-7 PD-L1 upregulation is independent of IFNγ. Mice were transcervically infected with 10^6 IFU of *C. trachomatis.* Five days post infection, dLN DCs (left graph) and uterine epithelial cells (right graph) were examined for PD-L1 expression by flow cytometry. Bar graphs show the average gMFI of PD-L1 from five mice per group. Error bars represent SEM. Statistical significance is indicated by *$p<0.05$ and **$p<0.01$ by Mann-Whitney Test.

The receptors for PD-L1 are upregulated during the memory response

PD-L1 has two described receptors: PD-1 and B7-1, both of which are expressed on T cells. Engagement of PD-L1 with either receptor on $CD8^+$ T cells can negatively regulate T cell function, and upregulation of PD-1 or B7-1 in memory $CD8^+$ T cells may be an indication of T cell exhaustion (4, 21-23). Although neither receptor was upregulated by qRT-PCR in *C. trachomatis* infected mice (**Figure 3-1**), it is possible that the qRT-PCR assay of the whole uterine tissue was not sensitive enough to detect subtle differences of PD-1 and B7-1 expression on specific cell subsets. Therefore, I measured PD-1 and B7-1 surface expression by flow cytometry on different cell subsets at time points following primary infection, during memory phase, and after secondary infection.

Neither PD-1 nor B7-1 displayed differences in expression in primarily infected mice compared to uninfected mice in any tissue I examined (**Figure 3-8**). However, during the memory response at 27 days following primary infection both PD-1 and B7-1 surface expression were significantly upregulated on $CD8^+$ T cells within the dLNs (**Figure 3-8**). B7-1 also displayed significant upregulation of surface expression on $CD8^+$ T cells in the dLNs after the secondary infection, whereas PD-1 expression returned to levels comparable to uninfected mice. As the expression of PD-1 and B7-1 are markers of $CD8^+$ T cell exhaustion, the upregulation of these molecules during memory phase suggests that $CD8^+$ memory T cells become exhausted following primary *C. trachomatis* transcervical infection.

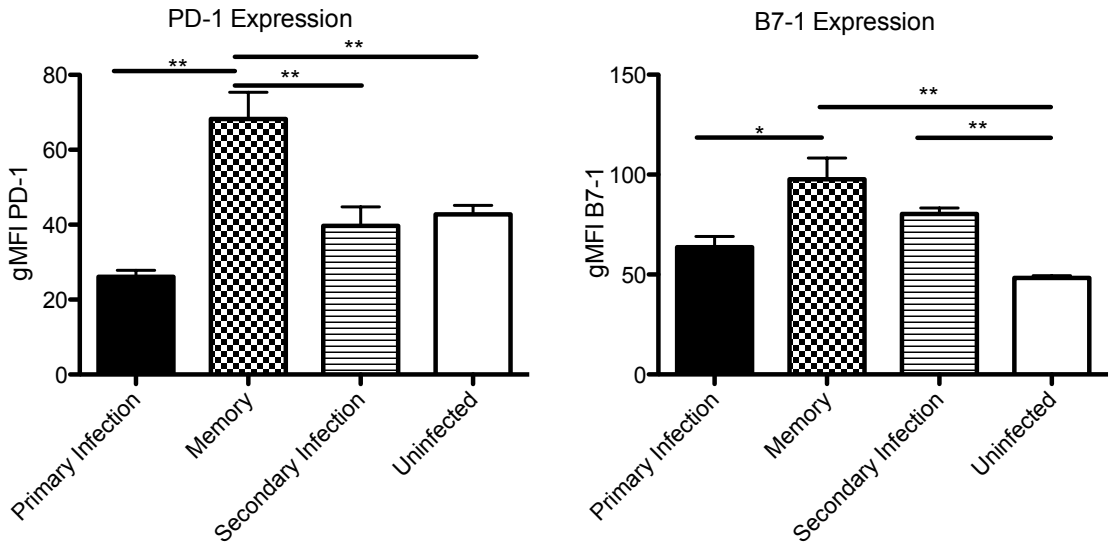

Figure 3-8 Surface expression of PD-L1 receptors, PD-1 and B7-1, is upregulated after *C. trachomatis* infection resolution. Mice were transcervically infected with 10^6 IFU of *C. trachomatis*. PD-1 (left) and B7-1 (right) expression was measured by flow cytometry on $CD8^+$ T cells in the dLNs five days post primary infection, 27 days post primary infection (memory), or five

(Figure 3-8 Continued)

days post secondary infection. Bar graphs show gMFI of PD-1 expression or B7-1 expression on $CD3^+$ $CD8^+$ T cells; shown is the average of 5 mice per time point and error bars represent SEM. Statistical significance is indicated by *$p<0.05$ and **$p<0.01$ by Mann-Whitney Test.

Taken together, these data demonstrate that PD-L1 expression is highly upregulated on uterine epithelial cells and the dLN DCs upon *C. trachomatis* infection. Furthermore, the receptors for PD-L1 are also upregulated on $CD8^+$ T cells once infection has cleared. This work provides evidence suggesting a role of PD-L1- mediated inhibition of the immune response during *C. trachomatis* infection of the uterus.

Discussion

During $CD8^+$ T cell activation, stimulatory and inhibitory molecules provide important signaling to the T cell that ultimately shapes the T cell response. Importantly there must be a balance between stimulatory and inhibitory signaling to produce an effective response that limits pathological damage. It was hypothesized that *C. trachomatis* infection would tip that balance toward inhibitory signaling, which could significantly impair the $CD8^+$ T cell response. This work sought to identify potential inhibitory pathways that could contribute to $CD8^+$ T cell dysfunction during *C. trachomatis* infection. I observed that PD-L1 expression was highly upregulated during *C. trachomatis* infection and that PD-L1 upregulation was specific to infection with productive *C. trachomatis* infection.

The role of PD-L1 in immune regulation has been well described during chronic viral infection. During HIV infection, PD-L1 is highly expressed on pAPCs and PD-1 expressing-$CD8^+$ T cells from HIV-infected patients have impaired proliferative and IFNγ production capacity (24, 25). Furthermore, there is a positive correlation between PD-1 expression on $CD8^+$ T cells and HIV disease progression, indicating that the PD-L1/PD-1 pathway may play an important role in limiting viral replication (24). Blockade of the PD-L1/PD-1 pathway results in expansion of HIV specific, IFNγ-producing $CD8^+$ T cells and suggests that this is a pathway that could be a viable therapeutic target (26).

The PD-1/PD-L1 pathway also impairs the $CD8^+$ T cell response to Hepatitis B Virus (HBV). PD-L1 is expressed on almost all cell types within a HBV inflamed mouse liver, and PD-1 is upregulated on HBV specific $CD8^+$ T cells (27). Similar to HIV

infection, blockade of the PD-1/PD-L1 pathway results in the expansion of IFNγ producing $CD8^+$ T cells (28). Blockade of this pathway reduces HBV persistence in mice (27, 28).

It is clear that PD-L1 expression impacts the immune response and pathogen clearance. However, *how* PD-L1 expression results in delayed viral clearance is not fully understood. PD-L1 expression on APCs can affect the development of $CD8^+$ T cells during priming and result in memory $CD8^+$ T cells that lose cytotoxic function; ultimately these impaired $CD8^+$ T cells cannot efficiently clear the pathogen. However, blockade of the PD-1/PD-L1 pathway after memory $CD8^+$ T cell development has already occurred can still improve viral clearance, indicating another mechanism by which the PD-1/PD-L1 pathway impairs immune function. Interestingly, different cell types expressing PD-L1 can differentially impact the immune response. For example, PD-L1 deficiency on hematopoietic cells results in improved expansion and cytokine production of $CD8^+$ T cells during LCMV-Cl13 infection, however this does not necessarily lead to enhanced viral clearance (13). PD-L1 deficiency on non-hematopoietic cells does lead to enhanced viral clearance as well as severe immunopathology (13). These studies suggest that PD-L1 expression on pAPCs can alter $CD8^+$ T cell responses, but PD-L1 expression on infected cells may protect these cells from cytolytic activity of $CD8^+$ T cells. It is interesting that during *C. trachomatis* infection, PD-L1 surface expression is upregulated on both dendritic cells and epithelial cells. This suggests that PD-L1 might have multiple roles during *C. trachomatis* infection. Chapter Four of this thesis will examine the possible roles of the PD-L1/PD-1 pathway during *C. trachomatis* infection.

Although the PD-L1/PD-1 pathway plays a significant role in impairing the immune response to chronic viral infections, the majority of the upregulation of PD-L1 is associated with persistent antigen presence and inflammation, and clearance of the antigen can result in a reduction of PD-L1 expression. Although *C. trachomatis* produces chronic infections in human, *C. trachomatis* produces an acute infection in mice. Therefore it was surprising to see that PD-L1 was so highly expressed in the murine genital tract during *C. trachomatis* infection. However, in this instance PD-L1 expression is dependent on the presence of infection, as it returned to pre-infection levels once infection is cleared. Additionally, unlike infections with certain viral pathogens, PD-L1 expression during *C. trachomatis* infection was independent of IFNγ expression.

What remains entirely unclear in the PD-L1/PD-1 field is whether a pathogen can directly alter PD-L1 or PD-1 expression for benefit of the pathogen. In many models it has been shown that PD-L1 upregulation is dependent on the expression of IFNγ (19, 29). In the case of *C. trachomatis,* the data suggest that *C. trachomatis* directly upregulates expression of PD-L1, rather than through innate IFNγ signaling. *C. trachomatis* has over 120 known and predicted type III secreted effectors, and future studies will aim to understand if one or more of these effectors could be involved in altering PD-L1 expression.

Like chronic viral infections, infection with *C. trachomatis* leads to the development of $CD8^+$ T cells that exhibit signs of exhaustion. Specifically, PD-1 expression on $CD8^+$ T cells is a widely used marker for T cell exhaustion. The data here indicate that both PD-1 and B7-1 are highly expressed on memory $CD8^+$ T cells following *C. trachomatis* infection, suggesting that these $CD8^+$ T cells may be exhausted.

Future studies will test if memory $CD8^+$ T cells from *C. trachomatis* infected mice are truly exhausted by measuring cytokine production and proliferation during *in vitro* stimulation. If $CD8^+$ T cells exhibit an impaired ability to produce IFNγ and IL-2 or to proliferate, this would indicate that $CD8^+$ T cells become exhausted following *C. trachomatis* infection. If $CD8^+$ T cells are in fact exhausted or impaired by interaction of PD-L1 with one or both of its receptors during *C. trachomatis* infection, it may explain the diminished $CD8^+$ T cell population during secondary *C. trachomatis* infection and the inability of $CD8^+$ T cells to contribute to controlling infection. These questions will be addressed in the next chapter.

Methods

Mice

C57BL/6 and B6.129S7-IFNγtm1Agt (IFNγ-/-) mice were purchased from The Jackson Labs. All animals were maintained and cared for within the Harvard Medical School Center for Animal Resources and Comparative Medicine (Boston, MA). All mice were treated with 2.5 mg medroxyprogesterone subcutaneously 7 days prior to infection with *C. trachomatis* to synchronize the murine estrous cycle. Mice were infected with 10μl of infectious agent using a non-surgical embryo transfer device (ParaTechs). All experiments were approved by the Institutional Animal Care and Use Committee.

Growth, isolation, and detection of bacteria

C. trachomatis serovar L2 (434/Bu) was propagated within McCoy cell monolayers grown in Eagle's MEM (Invitrogen, Grand Island, NY) supplemented with 10% FCS, 1.5 g/l sodium bicarbonate, 0.1M nonessential amino acids, and 1 mM sodium pyruvate. Infected monolayers were disassociated from plates using sterile glass beads and were sonicated to disrupt the inclusion. Elementary bodies were purified by density gradient centrifugation, as described previously (30, 31). Aliquots were stored at -80°C in medium containing 250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid and were thawed immediately prior to use. To quantify the levels of C. trachomatis, quantitative PCR (qPCR) with 16S primers specific for *C. trachomatis* was performed as has been previously described (30).

Flow cytometry

Tissues were mechanically disaggregated and immediately stained for surface markers. Cells were preincubated with anti-FcRg (Bio X-Cell) before staining with αCrpA-APC (National Institute of Health Tetramer Core) or αPD-L1-APC, αCD4 Q-Dot, αCD8-APC-Cy7, and αPD-1-PeCy7, αB7-1-PE, αCD90.2-PerCP (Biolegend). Cells were also incubated with αCD11b-PB and αCD11c-FITC. When necessary, the absolute cell number in each sample was determined using AccuCheck Counting Beads (Invitrogen). Data were collected on an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star).

Inhibitory gene transcript expression

Mice were transcervically infected with 10^6 inclusion forming units (IFU) of *C. trachomatis* as previously described (32). Five days after infection, tissues were mechanically disaggregated in 2 ml of PBS and aliquots immediately frozen at -20°C. RNA was extracted from 80µl aliquots by phenol-chloroform precipitation. Quantitative reverse transcriptase PCR (qRT-PCR) was performed using 25ng of purified RNA and amplified using Taqman SYBR Green mastermix. The following primers were used: CTLA4 Sense: 5′-GTTGGGGGCATTTTCACATA-3 CTLA4 Antisense: 5′-TTTTACAGTTTCCTGGTCTC-3; Tim3 Sense: 5′-GAACTGAAATTAGACATCAAAGCAGC-3′ Tim3 Antisense: 5′-GGTTCTTGGAGAAGCTGTAGTAGAGTC-3′; Lag3 Sense: 5′-TCCGCCTGCGCGTCG-3′, Lag3 Antisense: 5′-GACCCAATCAGACAGCTTGAGGAC-3′; CD160 Sense: 5-

GGCCACTTTCTCTCCGTTCTAG, CD160 Antisense: 5-

GGTGTGACCTTTGTCTCTGTCTTATC-3; 2B4 Sense: 5′-

GTTGCCACAGCAGACTTTC

2B4 Antisense: 3′-TTCCAACCTCCTCGTACACGGTAC; PD-1 Sense 5′:

CCCTCAGTCAAGAGGAGCAT; PD-1 Antisense 5′-TCCCAGCTTGTGGTAAACCT;

PD-L2 Sense 5′: GTACCGTTGCCTGGTCATCT

PD-L2 Antisense 5′: GCC AGG ACA CTT CTG CTA GG-3′; B7-1 Sense 5′-

ATGGCTTGCAATTGTCAGTTGA-3′

B7-1 Antisense 5′-ATCAGGAGGGTCTTCTGGGGG T-3′; PD-L1 Sense 5′-TGG ACA

AAC AGT GAC CAC CAA-3′, PD-L1 antisense 5′-CCC CTC TGT CCG GGA AGT-3′.

GAPDH Sense 5′-GGTGCTGAGTATGTCGTGGA-3′; GAPDH antisense 5′-

CGGAGATGATGACCCTTTTG-3

Statistical analysis

The data represent the mean ± SEM and were calculated using GraphPad Prism version 4.0. P values were determined using the Mann-Whitney Test or Students T-test. Significant differences between groups are indicated as follows: *$p<0.05$, **$p< 0.01$, and ***$p<0.005$.

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Chapter Four: PD-L1 limits the mucosal $CD8^+$ T cell response to *Chlamydia trachomatis*

PD-L1 limits the mucosal CD8$^+$ T cell response to *Chlamydia trachomatis*

Sarah Fankhauser and Michael Starnbach

Portions of this chapter have been submitted as part of a manuscript to the Journal of Immunology. The project was conceived by S. Fankhauser and M. Starnbach. Experiments and data analysis were performed by S. Fankhauser, and all text and figures were produced by S. Fankhauser.

Introduction

Chlamydia trachomatis is an obligate intracellular bacterial pathogen that infects 1.4 million people in the United States each year, and the incidence of infection has continued to rise since 2000 (1). Adolescent girls and young women infected with *C. trachomatis* face reproductive tract damage and increased risk of ectopic pregnancy and infertility (2). Importantly, long term immunity does not develop and the risk of reproductive damage increases with multiple *C. trachomatis* infections (3-6)

The work in previous chapters of this thesis detailed the defective $CD8^+$ T cell response to *C. trachomatis* that could contribute to the lack of, or impaired, long-term immunity that develops during infection. I hypothesized that a known immunoinhibitory pathway may be upregulated during infection and thus contribute to impairing the $CD8^+$ T cell response. PD-L1 expression is highly upregulated during *C. trachomatis* infection and therefore is a prime candidate for further investigation. Although PD-1 expression is typically associated with persistent antigen, it is not until *C. trachomatis* infection has cleared that PD-1 surface expression is upregulated on $CD8^+$ T cells. The expression of the other PD-L1 receptor, B7-1, also exhibited increased expression on $CD8^+$ T cells during memory phase. These data suggest that the $CD8^+$ T cells may become exhausted, and this may explain the blunted recall response of the $CD8^+$ T cell population after rechallenge with *C. trachomatis.*

It is PD-1 expression on $CD8^+$ T cells that is highly associated with an apoptotic phenotyope, and its interaction with PD-L1 has been well described in the literature (7, 8). During chronic viral infection with LCMV-Cl13, PD-L1-expressing professional

antigen presenting cells (pAPCs) engage PD-1 on $CD8^+$ T cells. The engagement of the PD-L1/PD-1 pathway antagonizes the T cell signaling mediated by stimulatory molecules, as well as affects downstream signaling pathways that decreases cytokine production and reduces memory potential (9, 10). The $CD8^+$ T cells that upregulate PD-1 exhibit a hierarchal loss of function, eventually becoming nonresponsive to antigen.

The other described receptor for PD-L1 is B7-1. B7-1 expressed on pAPCs engages CD28 on T cells to produce costimulatory signaling, or CTLA-4 on T cells to produce inhibitory signaling. B7-1 is also expressed on T cells and engages PD-L1 to produce inhibitory signaling. The role of PD-L1/B7-1 signaling is not as clear as PD-L1/PD-1 in pathogenic models but there is evidence that the PD-L1/B7-1 pathway partially restrains self-reactive $CD4^+$ and $CD8^+$ T cells in non-obese diabetic mice (11).

The work in this chapter describes the role of PD-L1 in inhibiting the $CD8^+$ T cell response to *C. trachomatis* and the implications this has on bacterial clearance. Here I show that the $CD8^+$ T cell response to genital infection with *C. trachomatis*, as with chronic viral infections, is negatively affected by the immunoinhibitory receptor PD-1 and its ligand, PD-L1. Deletion or inhibition of this pathway improves the $CD8^+$ T cell response and results in enhanced bacterial clearance.

Results

PD-L1 and PD-1 contribute to control of *C. trachomatis* burden in the uterus

The significant upregulation of PD-L1 expression in the genital tract upon *C. trachomatis* infection led me to consider that PD-L1 may impact the clearance of *C. trachomatis*. To test this, I examined bacterial burden by quantitative real time PCR (qPCR) in WT mice and PD-L1 deficient mice five days after primary transcervical infection. PD-L1 deficient mice exhibited a ten-fold lower bacterial load compared to WT mice (**Figure 4-1**). I also tested whether the loss of PD-L1 affected protection during secondary challenge. WT and PD-L1 deficient mice were transcervically infected with *C. trachomatis*, allowed to recover for four weeks and then reinfected transcervically. Bacterial burden was measured in the uterus five days following secondary infection. WT and PD-L1 deficient mice exhibited similar bacterial levels (**Figure 4-1**). These data demonstrate that loss of PD-L1 improves bacterial clearance during primary infection, but has no effect on the ability of the host to clear secondary infection.

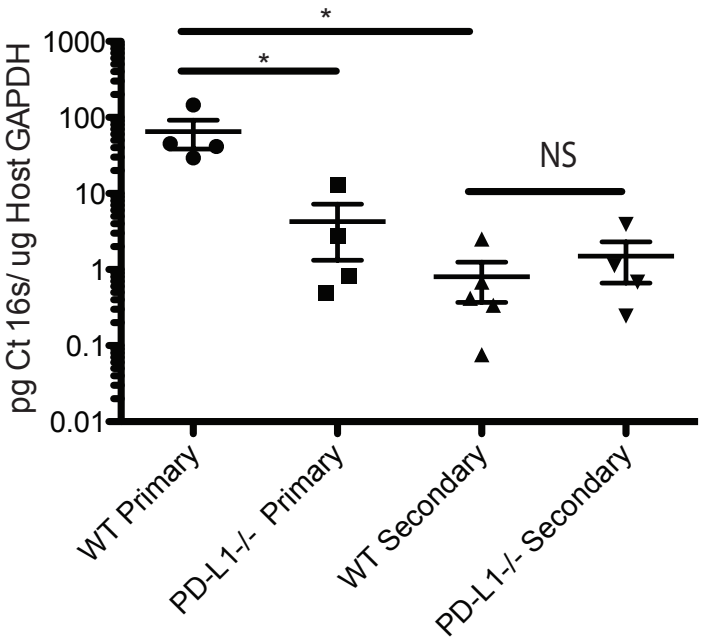

Figure 4-1 PD-L1 expression limits bacterial clearance. WT and PD-L1 deficient mice were transcervically infected with 10^6 IFU of *C. trachomatis*. Five days after primary and secondary infection, bacterial levels in the uterus were measured by qPCR and normalized to host GAPDH levels. Similar results were obtained in three independent experiments. Error bars represent SEM. *$p<0.05$ by the Mann-Whitney test.

Since PD-L1 deficient mice clear primary infection more efficiently, I sought to determine if therapeutically blocking PD-L1 would also enhance bacterial clearance. To test this, I transiently blocked PD-L1 by administering anti-PD-L1 antibody three days prior and two days after primary infection. Five days after primary infection I measured bacterial burden in the genital tracts of mice and observed that mice treated with anti-PD-L1 antibody showed over a two log reduction in bacterial burden compared to mice treated with isotype antibody (**Figure 4-2**). Similar to results observed in PD-L1 deficient mice, mice that were treated with anti-PD-L1 antibody prior to primary

infection and then subsequently reinfected four weeks later exhibited no difference in bacterial burden compared to mice treated with isotype antibody (**Figure 4-2**). These data indicate that transient blockade of PD-L1 during primary infection is sufficient to improve bacterial clearance, and does not affect bacterial burden during secondary infection. These data suggest that protection during secondary infection may be mediated by PD-L1-independent adaptive immune responses.

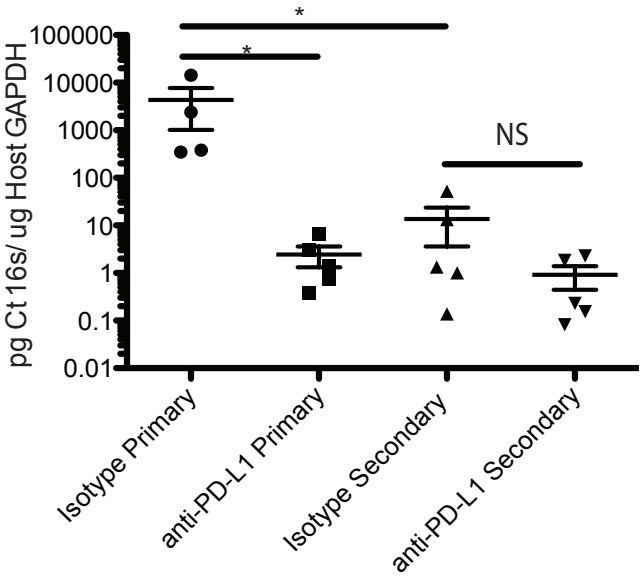

Figure 4-2 Transient PD-L1 inhibition enhances *C. trachomatis* clearance. WT mice were treated with isotype control or anti-PD-L1 antibody 3 days prior to and 2 days after primary transcervical infection with 10^6 IFU. Five days after primary or secondary infection bacterial levels were measured by qPCR and normalized to host GAPDH levels. Similar results were obtained in two independent experiments. Error bars represent SEM. *$p<0.05$ by the Mann-Whitney test.

PD-L1 can signal through the receptors PD-1 and B7-1. In order to test which receptor PD-L1 was acting through to limit efficient bacterial clearance, I compared bacterial levels in WT mice and PD-1 deficient mice five days after primary transcervical

infection. PD-1 deficient mice had significantly lower bacterial burden compared to WT (**Figure 4-3**). To determine if PD-L1 may also act through the receptor B7-1, I treated mice three days before and two days after primary infection with an antibody to block B7-1, or isotype control. Five days after transcervical infection I measured bacterial levels in the uterus and determined that there was no significant difference in bacterial burden in mice treated with anti-B7-1 antibody compared to mice treated with isotype control (**Figure 4-4**). Together these data demonstrate that PD-L1 likely signals through PD-1 and not B7-1 to inhibit bacterial clearance. Moreover blockade or deletion of the interaction between PD-L1 and the receptor PD-1 leads to enhanced bacterial clearance.

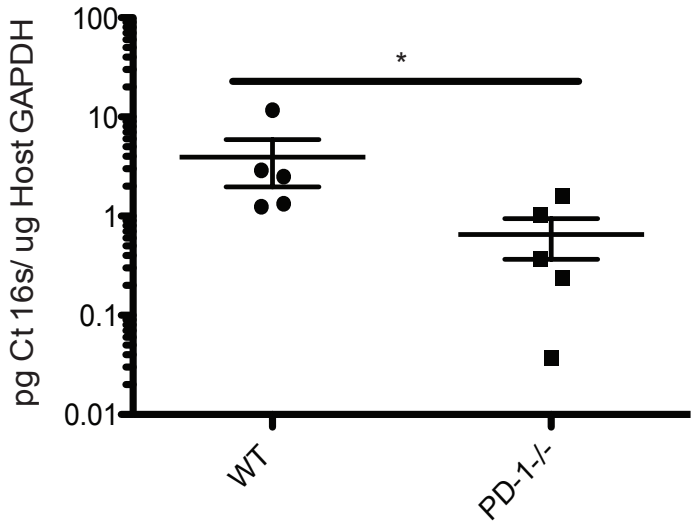

Figure 4-3 PD-1 expression limits *C. trachomatis* clearance. WT and PD-1 deficient mice were transcervically infected with 10^6 IFU. Five days after primary infection bacterial levels in the uterus were measured by qPCR and normalized to host GAPDH levels. Similar results were obtained in two different experiments. Error bars represent SEM. *$p<0.05$ by the Mann-Whitney test.

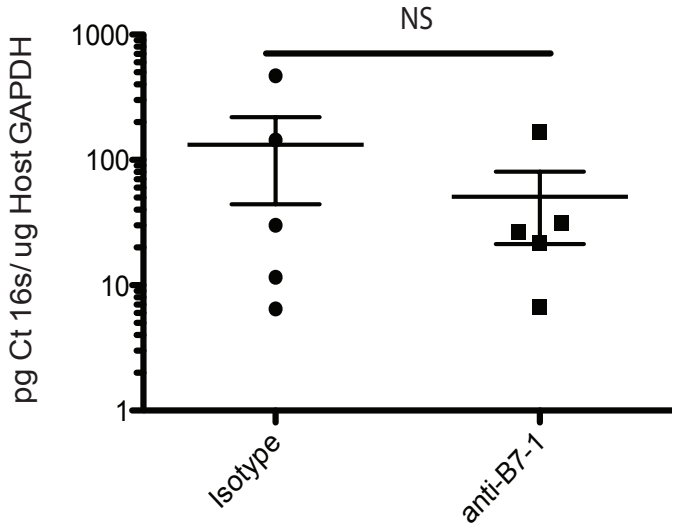

Figure 4-4 B7-1 does not contribute to *C. trachomatis* clearance. WT mice were treated with anti-B7-1 antibody or isotype control 3 days prior to and 2 days after transcervical infection with 10^6 IFU. Five days after primary infection the bacterial levels in the uterus were measured by qPCR and normalized to host GAPDH levels. Error bars represent SEM.

PD-L1 pathway contributes to the inhibition of the uterine CD8$^+$ T cell response following transcervical *C. trachomatis* infection

The experiments described in Chapter Three show that PD-L1 is expressed early during infection on dendritic cells in the draining lymph nodes (dLN DCs). DCs are the pAPCs that prime the CD8$^+$ T cells during *C. trachomatis* infection (12, 13). I therefore postulated that PD-L1 expression alters the CD8$^+$ T cell response that results in the defect in bacterial clearance I observed. To test this I assessed the phenotype of the CD8$^+$ T cells that developed during infection in WT and PD-L1 deficient mice. Memory CD8$^+$ T cells can be broadly separated into two subsets: central memory T cells (T_{cm}) and effector

memory T cells (T_{em}) (14, 15). These subsets are defined by the expression of the surface markers CD62L and CD127 (14-16). The lymph node homing marker, CD62L, mediates $CD8^+$ T cells extravasation into the vasculature and entry into lymphoid organs and is highly expressed on T_{cm} cells and naïve $CD8^+$ T cells (T_n) (15). T_{em} cells express low levels of CD62L which allows this population to stay in the periphery (15). The IL-7 receptor, CD127, is necessary for $CD8^+$ T cells to respond to IL-7 which promotes T cell development and persistence in the periphery. CD127 is highly expressed on both $T_{cm/n}$ and T_{em} cell subsets (14). To test if PD-L1 expression affects the $CD8^+$ T cell phenotype during *C. trachomatis* infection, I transcervically infected WT and PD-L1 deficient mice and compared the $T_{em}/T_{cm/n}$ ratio of the $CD8^+$ T cell response by flow cytometry following primary, during memory phase, and after secondary infection. At all three time points I observed a significant decrease in the percentage of $T_{cm/n}$ cells (CD62L high/CD127 high) in the genital tracts of PD-L1 deficient mice compared to WT mice (**Figures 4-5 A and 4-5B, left panel**). Additionally there was a slight but significant increase in the percentage of T_{em} cells (CD62L low/CD127 high) in the uteri of PD-L1 deficient mice (**Figures 4-5A and 4-5B, left panel**). When I measured total numbers of T_{em} and $T_{cm/n}$ cells in the genital tracts there was a significant three fold increase in the ratio of T_{em} to $T_{cm/n}$ cells in PD-L1 deficient mice, compared to WT mice during primary infection (**Figure 4-5C**). The three-fold increase in the ratio of T_{em} to $T_{cm/n}$ cells was consistent when I compared PD-L1 deficient mice to WT mice during the memory phase (**Figures 4-5A, 4-5B, and 4-5C middle panel**). Although there was an increase in the T_{em} to $T_{cm/n}$ ratio following secondary infection, this difference was not significant (**Figure 4-C left panel**). Notably, I was able to recapitulate the shift to a T_{em} phenotype

at all time points in PD-1 deficient mice (**Appendix B, Figure B-2**). These data indicate that PD-L1 expression skews the $CD8^+$ T cells towards a central memory phenotype during a primary *C. trachomatis* infection.

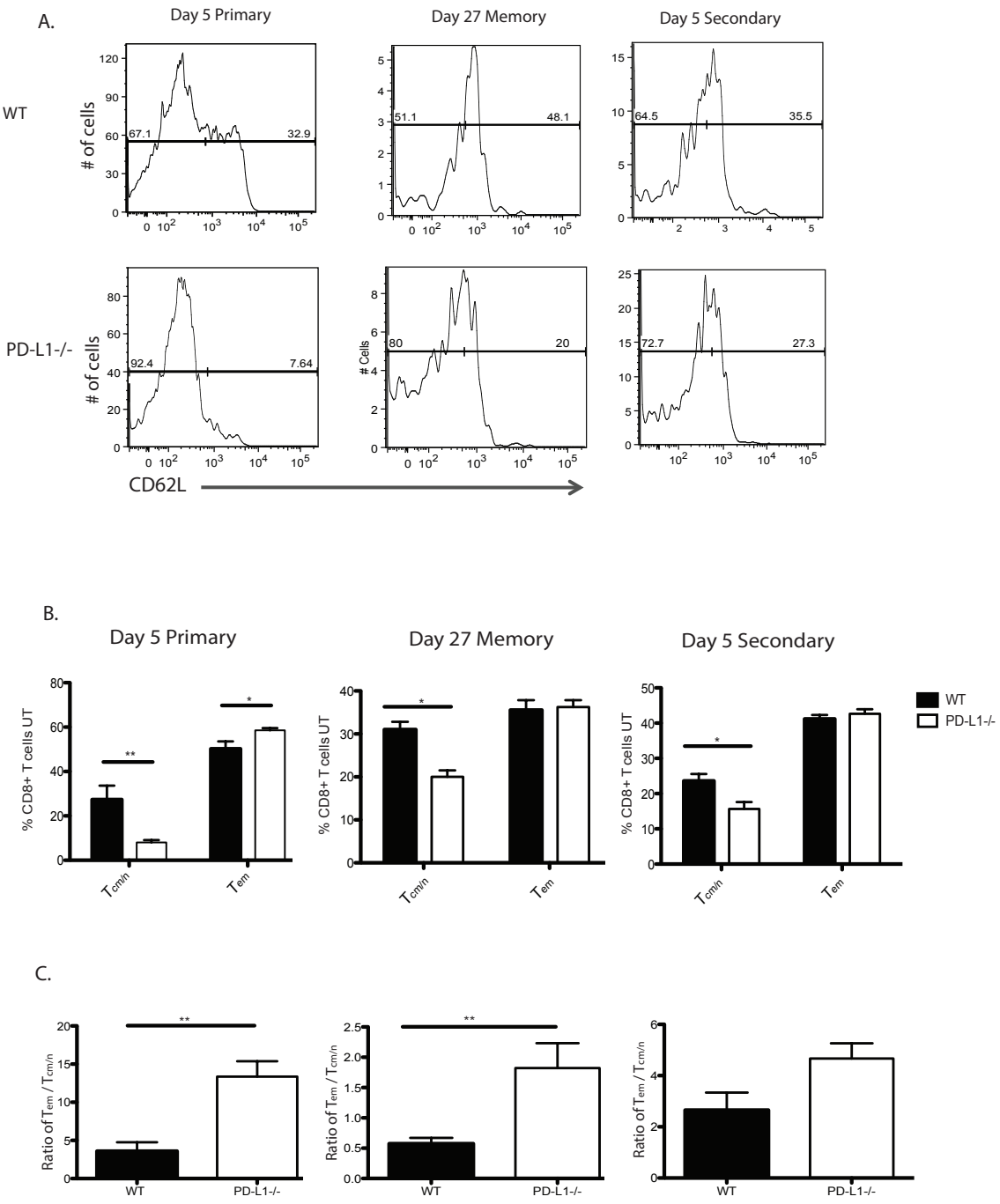

Figure 4-5 PD-L1 expression shifts the ratio of T_{em} and $T_{cm/n}$ $CD8^+$ T cells during *C. trachomatis* infection. (A) WT and PD-L1 deficient mice were transcervically infected with 10^6 IFU of *C. trachomatis*. At indicated

(Figure 4-5 Continued)

timepoints, uteri were harvested and stained for CD62L and CD127 expression. $CD8^+$ memory populations were gated on live, $CD90.2^+$, $CD127^+$, $CD4^-$, $CD8^+$ cells; histograms of CD62L expression of this population are shown. (B) Quantification of uterine T_{em} cells (CD127 high/CD62L low) and $T_{cm/n}$ (CD127 high/CD62L high) cells at time points indicated in WT and PD-L1 deficient mice. Bars represent mean of 4-5 mice per group. (C) Ratio of the absolute numbers of T_{em} to $T_{cm/n}$ $CD8^+$ T cells found in genital tracts of WT and PD-L1 deficient mice. Bars represent mean ratios of 4-5 mice per group and error bars represent SEM. *$p<0.05$, **$p<0.01$ using the Mann-Whitney test.

To test whether early PD-L1 expression skews the priming of $CD8^+$ T cells toward an effector memory $CD8^+$ T cell response, I treated mice with an anti-PD-L1 antibody three days prior to and two days after primary infection. These mice were allowed to recover for 3 weeks before secondary infection in the absence of further antibody treatment. Five days after secondary infection, mice treated with the anti-PD-L1 antibody during primary infection had a significant increase in the ratio of T_{em} to $T_{cm/n}$ cells found in the genital tract compared to mice treated with isotype control (**Figure 4-6**). This is consistent with the phenotype I observed in PD-L1 deficient mice and suggests that transient inhibition of the PD-1/PD-L1 pathway during primary expansion is sufficient to shift the balance between T_{em} and $T_{cm/n}$ cells as they develop. Furthermore, this shift in T cell phenotype ratio is maintained through the recall response.

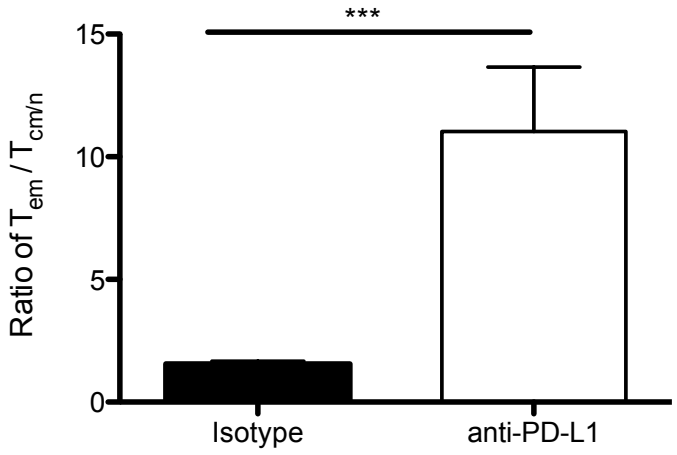

Figure 4-6 Inhibition of PD-L1 during primary infection alters the CD8$^+$ T cell phenotype during recall. Mice were treated with anti-PD-L1 blocking antibody or isotype antibody control three days prior and two days after primary transcervical infection with 10^6 IFU. Mice were allowed to recover for four weeks, at which point they were rechallenged transcervically with 10^6 IFU of *C. trachomatis*. Five days after rechallenge, the number of effector and central memory CD8$^+$ T cells were determined in the genital tract. Indicated is the ratio of effector to central memory CD8$^+$ T cells. ***$p<0.005$ by the Mann-Whitney test

Next I tested whether the shift towards the CD8$^+$ T cell effector memory phenotype in PD-L1 deficient mice would affect functions such as antigen-specificT cell abundance or IFNγ production. First I assessed whether the absence PD-L1 would restore the magnitude of the secondary CD8$^+$ T cell response that is lacking in WT *C. trachomatis* infected mice. Five days after secondary infection, I observed a significant 1.5 fold increase in the number of *C. trachomatis*-specific CD8$^+$ T cells found in the genital tract of PD-L1 deficient mice compared to WT mice (**Figure 4-7**). Moreover, I observed that the number of *C. trachomatis*-specific CD8$^+$ T cells in PD-L1 deficient mice was significantly higher in secondary infection compared to primary infection of PD-L1 deficient mice (**Figure 4-7**). These data suggest that the absence of PD-L1

signaling results in $CD8^+$ T cells that have enhanced recruitment to the genital tract, or are able to expand to greater numbers, upon reinfection.

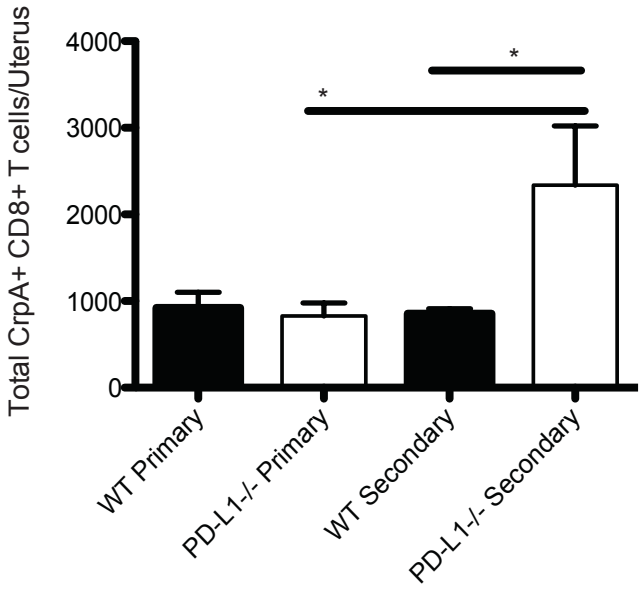

Figure 4-7 *C. trachomatis* specific $CD8^+$ T cell response is enhanced in PD-L1 deficient mice. Five days after primary or secondary transcervical challenge, the number of *Chlamydia*-specific $CD8^+$ T cells using a tetramer specific for the *Chlamydia* antigen CrpA were quantified in the genital tracts of WT and PD-L1 deficient mice. Similar results were obtained in two independent experiments. Bars represent the mean of 4-5 mice per group with SEM. *$p<0.05$ by the Mann-Whitney test.

The second functional consequence of PD-L1 deficiency I assessed was the expression of IFNγ, a cytokine critical in restricting *C. trachomatis* growth. I compared the ability of endogenous uterine $CD8^+$ T cells to produce IFNγ in WT and PD-L1 deficient mice. In comparison to WT mice I observed that PD-L1 deficient animals had a significant increase in the number of IFNγ-producing $CD8^+$ T cells by flow cytometry

during both primary and secondary responses (**Figure 4-8**). This suggests that PD-L1 deficiency leads to the expansion or retention of a larger population of $CD8^+$ T cells capable of producing IFNγ during both primary and secondary infection.

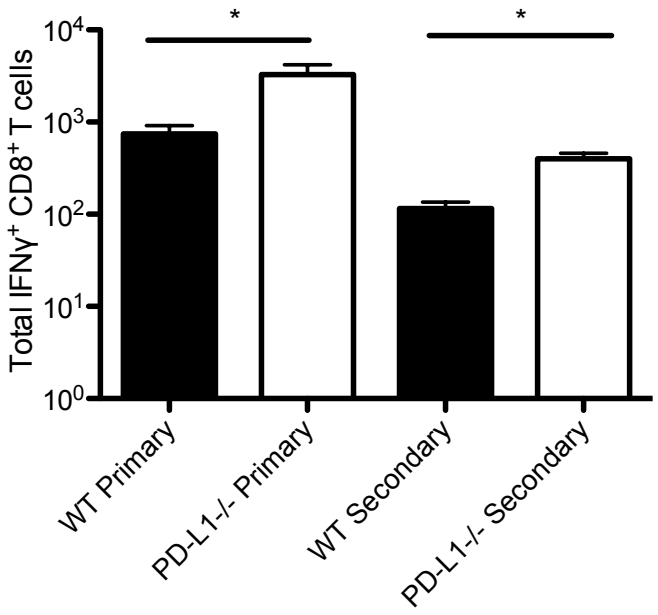

Figure 4-8 The $CD8^+$ IFNγ response is enhanced during *C. trahomatis* infection in PD-L1 deficient mice. Five days after primary or secondary transcervical challenge, the number of $IFN\gamma^+$ $CD8^+$ T cells in the uterus was measured by ICCS. Bars indicate the mean of five mice per group and error bars represent SEM. *$p<0.05$ by the Mann-Whitney test.

PD-L1 deficiency leads to a T_{em} skewed $CD8^+$ T cell population and an increased number of IFNγ producing $CD8^+$ T cells. However, it was unclear if this phenotype was the result of a decrease in cell death or an expansion of the T_{em} population. Previous reports indicated that PD-L1 expression led to the increased expression of the pro-apoptotic marker, Bim, which subsequently limited the number of $CD8^+$ T cells in the memory pool (17). To test if PD-L1 expression differentially effected Bim expression in

$CD8^+$ T cell populations, I examined the expression of Bim during primary *C. trachomatis* infection of mice treated with anti-PD-L1 or control antibody. Five days after primary infection I examined Bim expression by flow cytometry in different T cell populations. Bim expression was similar in $CD4^+$ T cells from mice treated with control and anti-PD-L1 antibodies, however Bim expression was significantly lower in $CD8^+$ T cells from mice treated with anti-PD-L1 antibody (**Figure 4-9 A and B**). When I separated $CD8^+$ T cells into $T_{cm/n}$ and T_{em} populations I observed that Bim expression did not significantly differ in $T_{cm/n}$ cells from mice treated with control antibody or anti-PD-L1 antibody (**Figure 4-9 C**). However, Bim expression was significantly lower in T_{em} cells of mice treated with anti-PD-L1 antibody, compared to mice treated with control antibody (**Figure 4-9 D**). These results suggest that PD-L1 selectively increases Bim expression in T_{em} cells, likely contributing to the decrease in this population. Although Bim-mediated apoptosis of T_{em} cells may be one mechanism that PD-L1 expression limits this population, it does not exclude the possibilities that PD-L1 may also limit expansion potential or recruitment of specific populations to the uterus.

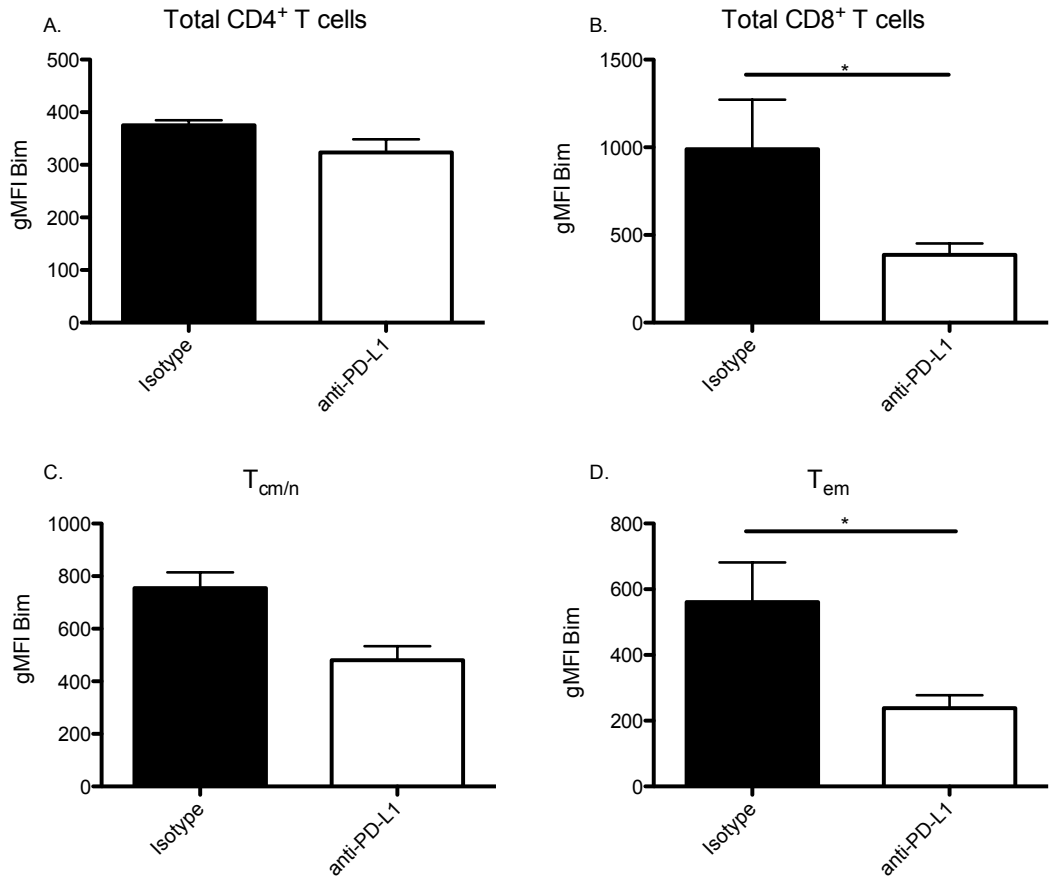

Figure 4-9 Bim expression is lower upon PD-L1 blockade. Mice were treated with isotype control or anti-PD-L1 blocking antibody three days prior and two days after primary transcervical infection with 10^6 IFU. Five days after infection, Bim expression was measured in $CD4^+$ T cells (A), $CD8^+$ T cells (B), $T_{cm/n}$ $CD8^+$ T cells (C) and T_{em} $CD8^+$ T cells (D) from the genital tracts. Shown is the average geometric mean fluorescent intensity (gMFI) of 4-5 mice per group. *$p<0.05$ by the Mann-Whitney test.

In the absence of PD-L1 $CD8^+$ T cells contribute to protection against *C. trachomatis*.

The shift to an effector memory $CD8^+$ T cell response in PD-L1 deficient mice led me to question whether the $CD8^+$ T cell population was now able to contribute to limiting

bacterial burden. To test this, I administered anti-$CD8^+$ antibody or isotype antibody to PD-L1 deficient and WT mice three days before and two days after primary infection. I confirmed by flow cytometry a 10-100-fold decrease in $CD8^+$ T cells in the uterus, dLNs, and spleen of mice treated with the anti-CD8 antibody (**Appendix B, Figure B-6**). Five days after infection I measured bacterial levels. $CD8^+$ T cell depletion in WT mice did not exacerbate bacterial burden compared to WT mice treated with control antibody (**Figure 4-10**). As expected, PD-L1 deficient mice treated with control antibody showed significantly lower bacterial burden compared to WT mice treated with control antibody (**Figure 4-10**). However, PD-L1 deficient mice treated with anti-CD8 antibody no longer exhibited the lower bacterial levels observed in PD-L1 deficient mice treated with control antibody (**Figure 4-10**). Although $CD8^+$ T cells appear to be dispensable in WT mice, PD-L1 deficient mice require $CD8^+$ T cells to confer total enhanced bacterial clearance. These data therefore support that the enhanced bacterial clearance seen in PD-L1 deficient mice is in part mediated by the altered $CD8^+$ T cell response.

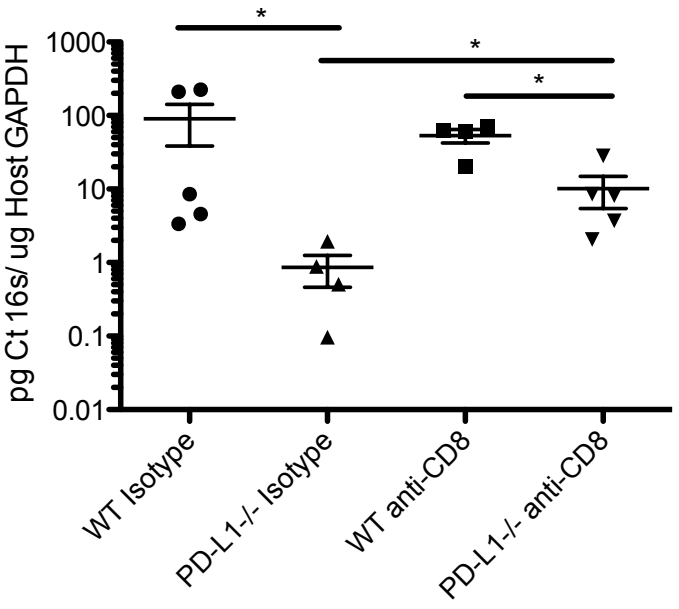

Figure 4-10 CD8$^+$ T cells contribute to bacterial clearance in PD-L1 deficient mice. WT and PD-L1 deficient mice were treated with anti-CD8 depleting antibody or isotype control antibody 3 days prior to and 2 days after primary infection. Mice were infected with 10^6 IFU of *C. trachomatis*, and five days post infection bacterial burden was measured in the uterus by qPCR and normalized to host GAPDH levels. Similar results were obtained in 2 independent experiments. *p<0.05 by the Mann-Whitney test

Although the CD8$^+$ T cell recall response we observed (**Figures 4-6 and 4-7**) could potentially contribute to bacterial clearance, PD-L1 deficiency did not lead to enhanced bacterial clearance during secondary infection. I reasoned that it was possible that any secondary protection afforded by the enhanced CD8$^+$ T cell response in PD-L1 deficient mice would be masked by the even more efficient CD4$^+$ T cell response. To decouple secondary protection mediated by CD4$^+$ T cells from protection via CD8$^+$ T cells, I depleted CD4$^+$ T cells prior to secondary challenge in WT and PD-L1 deficient mice. Mice were transcervically infected and examined for bacterial burden and T cell

counts five days later. $CD4^+$ T cell depletion was confirmed by flow cytometry (**Appendix B, Figure B-7**). As expected, depletion of the $CD4^+$ T cells in WT mice led to significant exacerbated bacterial burden (**Figure 4-11**). However, $CD4^+$ T cell depletion in PD-L1 deficient mice did not lead to increased bacterial burden as in WT mice, and in fact there were significantly lower bacterial levels in PD-L1 deficient mice treated with anti-CD4 depleting antibody compared to WT mice treated with anti-CD4 depleting antibody (**Figure 4-11**). Thus, $CD4^+$ T cells are not necessary for protection against secondary challenge in PD-L1 deficient mice.

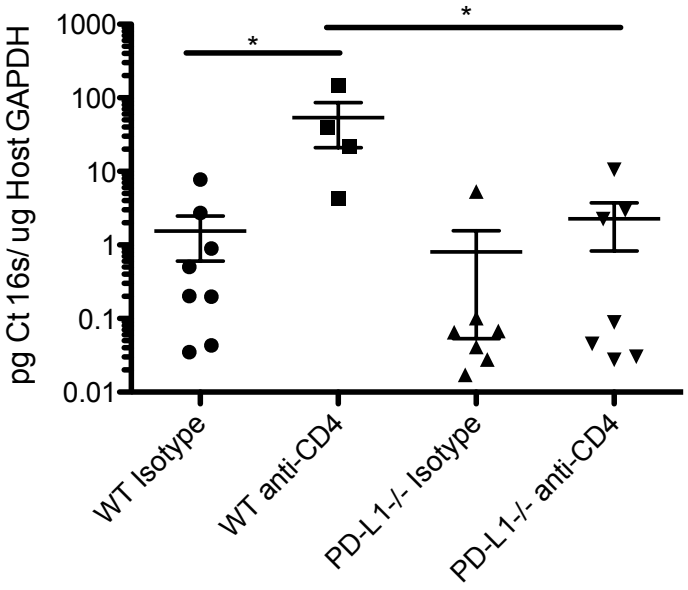

Figure 4-11 $CD4^+$ T cells are not required for protection in PD-L1 deficient mice. WT and PD-L1 deficient mice were treated with isotype control antibody or anti-CD4 antibody 3 days prior to and 2 days after secondary infection. Five days after secondary infection, bacterial levels were measured in the uterus by qPCR and normalized to host GAPDH levels. Shown in the compilation of 2 separate experiments *$p<0.05$ by the Mann-Whitney test

These data suggest that $CD8^+$ T cells can compensate for the lack of $CD4^+$ T cells during secondary infection. I next hypothesized that the altered phenotype of $CD8^+$ T cells, and not simply an increase in *C. trachomatis* specific $CD8^+$ T cells, secures protection in PD-L1 deficient mice. In order to determine whether $CD8^+$ T cells from WT and PD-1 deficient mice were different in their ability to protect mice against infection, I collected $CD8^+$ T cells from the spleen, lymph nodes and genital tracts of naïve WT and PD-1 deficient mice (**Figure 4-12 left panel**). I then transferred equal numbers of purified $CD8^+$ T cells into groups of naïve congenic IFNγ knockout mice, so that the only source of IFNγ was from the transferred $CD8^+$ T cells. One day after $CD8^+$ T cell transfer, I transcervically infected recipient mice with *C. trachomatis* (**Figure 4-12**). Five days after infection I measured the quantity of $CD8^+$ T cells in the dLNs and uteri. Both WT and PD-1 deficient $CD8^+$ T cells were able to migrate to the uterus; furthermore there were equal numbers of CrpA specific $CD8^+$ T cells in recipient mice (**Figure 4-13 a and b**). However, $CD8^+$ T cells from WT or PD-1 deficient mice were unable to confer protection compared to mice that received no T cells (**Figure 4-14**).

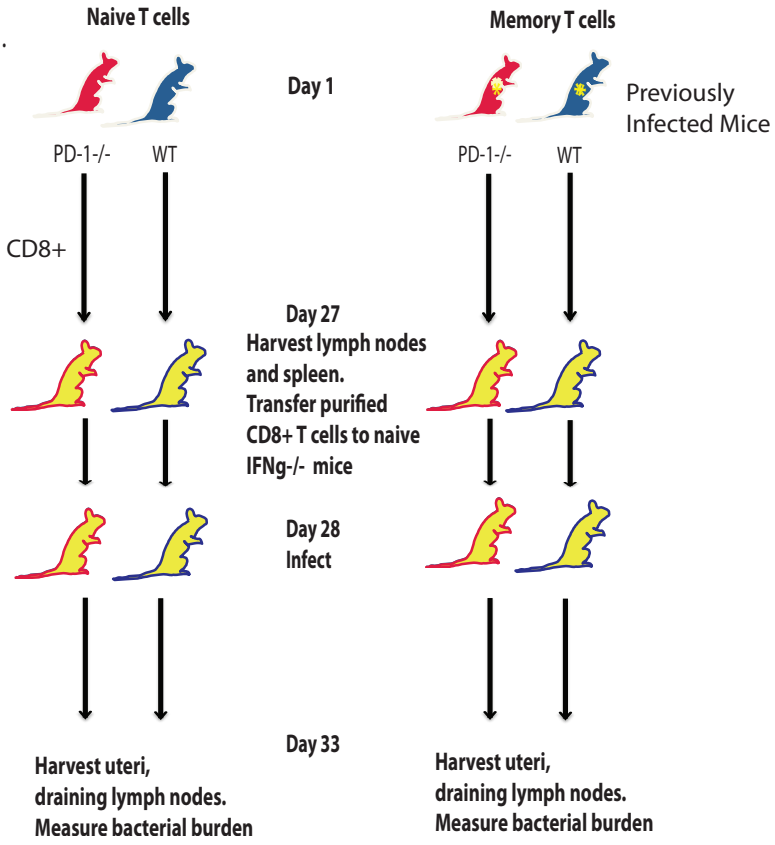

Figure 4-12 Experimental design of the T cell transfer experiment. $CD8^+$ T cells were isolated from naïve (left side) or previously *C. trachomatis* infected (right side) WT and PD-1 deficient mice. Isolated $CD8^+$ T cells were then transferred to naïve IFNγ knockout mice. One day after T cell transfer, recipient mice were transcervically infected with 10^6 IFU of *C. trachomatis*. Five days post infection, bacterial burden and T cell recruitment were measured.

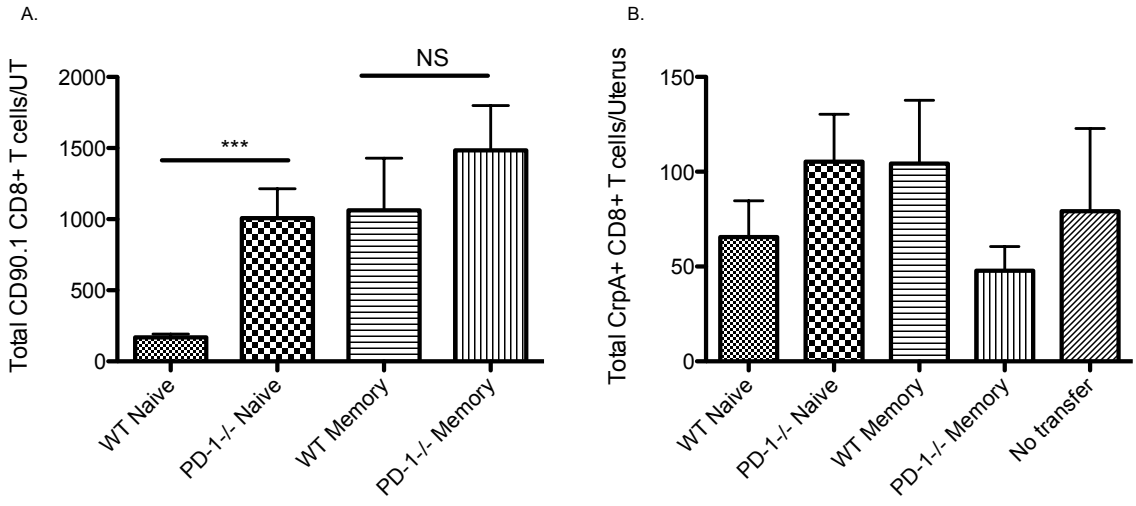

Figure 4-13 CD8$^+$ T cells are recruited to the genital tracts of naïve IFNγ recipient mice. A. Transferred CD8$^+$ T cell recruitment was measured in the genital tracts of recipient mice five days after transcervical infection; "naïve" and "memory" labels refer to origin of CD8$^+$ T cells. B. The total number of *C. trachomatis*-specific CD8$^+$ T cells were measured in the genital tract using tetramer specific for CrpA. Bars represent the mean of 6-8 mice per group and error bars indicate SEM. ***p<0.005 by the Mann-Whitney test.

Naïve WT and PD-1 deficient CD8$^+$ T cells start off equal in their abilities to limit *C. trachomatis* infection. I was curious if memory WT and PD-1 deficient CD8$^+$ T cells, primed during *C . trachomatis* infection differed in their capacity to limit *C. trachomatis* infection. To test this, I infected WT and PD-1 deficient mice and allowed these mice to recover for 4 weeks (**Figure 4-12, right side**). I then isolated CD8$^+$ T cells from these mice and transferred equal numbers of purified CD8$^+$ T cells to naïve IFNγ knockout hosts. One day later I infected recipient mice and five days after infection I measured the CD8$^+$ T cell response as well as bacterial burden in the uterus. I recovered almost equal numbers of WT and PD-1 deficient CD8$^+$ T cells in the uterus and draining lymph

nodes of recipient mice (**Figure 4-13a**). I also recovered similar numbers of *C. trachomatis* specific CrpA $CD8^+$ T cells in the uterus (**Figure 4-13b**.) When I measured bacterial levels in the genital tract of infected recipient mice I found that mice that received $CD8^+$ T cells from previously infected WT mice had bacterial levels similar to mice that received no T cells. However, mice that received $CD8^+$ T cells from previously infected PD-1 deficient mice had significantly lower bacterial levels compared to mice that received WT $CD8^+$ T cells (**Figure 4-14**). Taken together this data provide significant evidence that *C. trachomatis* infection severely impairs the $CD8^+$ T cell response via the PD-1/PD-L1 immunoinhibitory pathway, and disruption of the PD-1/PD-L1 pathway restores the protective capacity of these T cells.

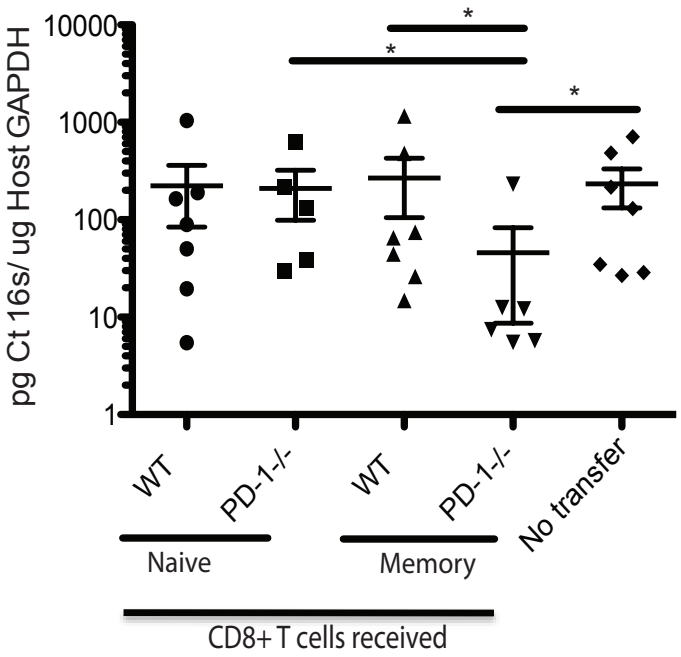

Figure 4-14 PD-1 deficient antigen-experienced CD8$^+$ T cells confer protection against *C. trachomatis* infection. Recipient IFNγ knockout mice were infected with 10^6 IFU of *C. trachomatis.* "Naïve" and "Memory" labels refer to origin of CD8$^+$ T cells. Five days after infection bacterial levels were measured in the uterus by qPCR and normalized to host GAPDH levels. Shown is the mean of 6-8 mice per group and error bars indicate SEM. Similar results were obtained in two independent experiments. *$p<0.05$ by the Mann-Whitney test.

PD-L1 is required on hematopoietic and non-hematopoietic cells to inhibit protection against *C. trachomatis*.

PD-L1 can be expressed on a variety of cell types, including DCs, T cells, endothelial cells and epithelial cells (18-20). I sought to determine which cellular population expressing PD-L1 influenced the shift in the CD8$^+$ T cell response to a more central memory response. It seemed likely that that PD-L1 expression on APCs would be required to bias the CD8$^+$ T cell response towards the central memory phenotype during

priming in WT mice. To test this, I created reciprocal bone-marrow chimeras from WT and PD-L1 deficient mice. After ensuring successful chimerism of at least 96%, I infected mice transcervically and analyzed the $CD8^+$ T cell response five days later. As expected, PD-L1 deficient mice that received PD-L1 deficient bone marrow had a significantly higher ratio of T_{em} to $T_{cm/n}$ cells found in the uterus, compared to WT mice that received WT bone marrow (**Figure 4-15**). WT mice that received PD-L1 deficient bone marrow also had a significantly higher ratio of T_{em} to $T_{cm/n}$ cells compared to WT mice that received WT bone marrow. However, the shift towards an effector memory phenotype was still significantly lower than that observed in PD-L1 deficient mice that received PD-L1 deficient bone marrow (**Figure 4-15**). PD-L1 deficient mice that received WT bone marrow had a similar ratio of T_{em} to $T_{cm/n}$ cells in the uterus compared to WT mice that received WT bone marrow (**Figure 4-15**). Overall these data indicate that PD-L1 expression on hematopoietic cells largely influences the $CD8^+$ T cell response and drives $CD8^+$ T cells to a more central memory phenotype during *C. trachomatis* infection. However, the absence of PD-L1 is required on both hematopoietic and non-hematopoietic cells to fully recapitulate the $CD8^+$ T cell response observed in PD-L1 deficient mice.

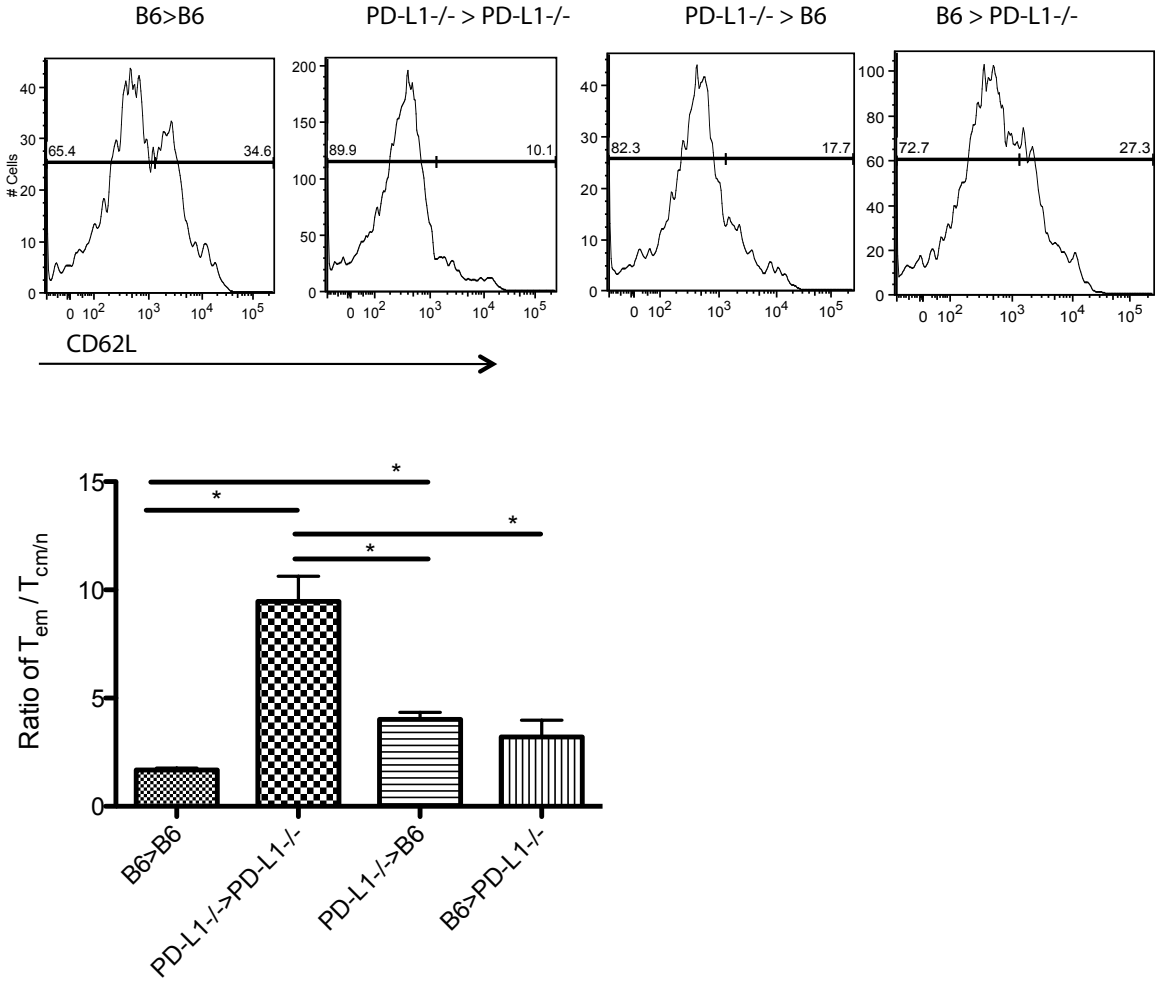

Figure 4-15 PD-L1 expression on hematopoietic and non-hematopoietic cells contributes to PD-L1 deficiency phenotype. WT and PD-L1 deficient reciprocal chimeric mice were transcervically infected with 10^6 IFU of *C. trachomatis*. The uteri were harvested five days after infection. The memory $CD8^+$ population was gated on live, $CD90^+$, $CD4^-$ $CD127^+$, $CD8^+$ T cells. Shown above is the histogram of CD62L expression of that population. Below is the ratio of T_{em} to $T_{cm/n}$ cells found in the uteri of chimera mice. Bars indicate the mean of 4-5 mice per group and error bars represent SEM. *$p<0.05$ by the Mann-Whitney test.

The absence of PD-L1 expression on hematopoietic cells alone was able to promote a $CD8^+$ T cell response that closely resembled that of PD-L1 deficient mice, however it was unclear if it was sufficient to decrease bacterial burden. Therefore I measured bacterial burden in the chimeric mice 5 days after infection. As shown in **Figure 4-16**, the absence of PD-L1 on either hematopoietic or non-hematopoietic cells alone was not enough to confer protection against *C. trachomatis*. These data therefore suggest that the expression of PD-L1 on both hematopoietic and non-hematopoietic cells contributes to limiting *C. trachomatis* clearance.

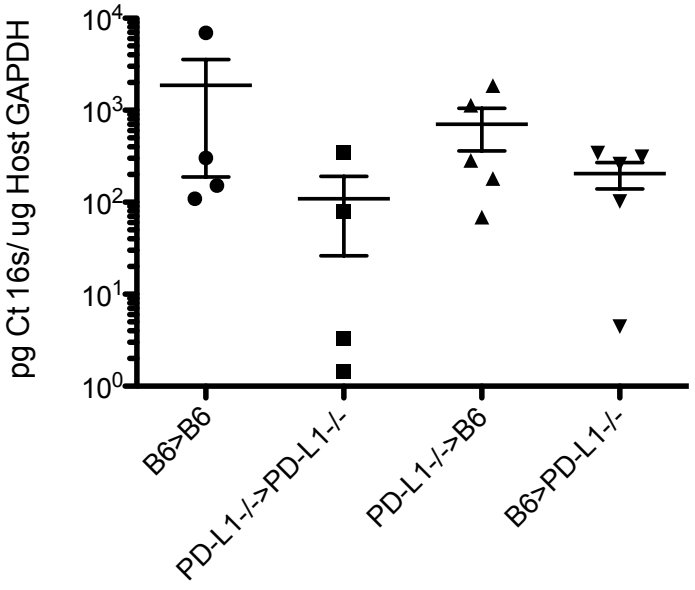

Figure 4-16 PD-L1 expression on hematopoietic and non-hematopoietic cells contributes to limiting *C. trachomatis* clearance. WT and PD-L1 deficient reciprocal chimeric mice were transcervically infected with 10^6 IFU of *C. trachomatis*. Five days after infection bacterial levels were measured in the uterus by qPCR and normalized to host GAPDH levels. Error bars represent SEM.

Discussion

Here I sought to identify mechanisms that contribute to the defective $CD8^+$ T cell response to *C. trachomatis*. I hypothesized that one or more inhibitory mechanisms may be upregulated during infection that would affect the quality of the $CD8^+$ T cell response. In the previous chapter I demonstrated that PD-L1 is strongly upregulated during *C. trachomatis* infection. Because PD-L1 has a well-known role in T cell exhaustion, I tested whether PD-L1 affects the $CD8^+$ T cell response during *C. trachomatis* infection. The data here demonstrate that PD-L1 expression alters the $CD8^+$ T cell memory phenotype, impairs the effector function of $CD8^+$ T cells and limits efficient *C. trachomatis* clearance.

The PD-1/PD-L1 pathway is a critical T cell regulatory pathway that is important in maintaining the balance between immune tolerance and effector function. In some pathogen models, engaging PD-1 on T cells has detrimental effects on pathogen elimination. For example, infection with the lower respiratory viruses Influenza A virus (IAV) or metapneumovirus induces PD-L1 expression on lung epithelial cells which leads to $CD8^+$ T cell impairment (21). Blockade of PD-L1 reverses $CD8^+$ T cell impairment, improves IFNγ production, and enhances viral clearance (21). In cancer models, contact of $CD8^+$ T cells with PD-$L1^+$ tumor cells results in a reduction of the cytolytic activity of the $CD8^+$ T cells and an impaired ability to clear the tumor cells (22). For bacterial pathogens, the role of PD-1/PD-1 is less clear. PD-L1 blockade in mice infected with *Listeria monocytogenes* impairs the $CD8^+$ T cell response and leads to exacerbated bacterial burden, indicating that PD-L1 acts as a stimulatory signal in this

context (23, 24). However, blockade of PD-L1 on monocytes infected with *Staphylococcus aureus* improves IL-2 production by $CD8^+$ T cells (25). Interestingly, my data indicate that blockade of PD-L1 improves the IFNγ response of $CD8^+$ T cells during *C. trachomatis* infection, leading to the enhanced bacterial clearance that is observed during primary infection.

A major source of variability in previous studies has been the anatomical site of infection, which may give rise to the diversity of effects attributed to the PD-1/PD-L1 pathway. Some anatomic sites need protection from host immune responses, and inhibitory mechanisms may be necessary to limit inflammation damaging to the host. In the respiratory tract for example, inhibitory PD-L1 signaling properly restricts T cells to avoid inflammation that could damage the lungs (26). The upper genital tract is also a privileged immune site since excessive inflammation could reduce fertility. During *C. trachomatis* infection, PD-L1 expression may allow the host to avoid an exaggerated and damaging $CD8^+$ T cell response. In fact, evidence suggests that $CD8^+$ T cells contribute to the uterine pathology that results from *C. trachomatis* infection (27, 28). One recent study demonstrates that blockade of PD-L1 in combination with the inhibitory receptor, Tim3, led to enhances uterine pathology of mice infected with *Chlamydia muridarum* (29). Although, it was unclear from that study what factors mediate the enhanced pathology, it provides evidence that immune-inhibitory pathways are important for limiting inflammation and pathology in the genital tract. Additional in-depth pathology studies will be required to understand if the lack of PD-L1 alone leads to enhanced pathology in the genital tract during *C. trachomatis* infection. It is possible that multiple inhibitory pathways are engaged during *C. trachomatis* infection in order to avoid

inflammation in the host, and deleting one of these pathways may not be sufficient to observe changes in gross pathology. We did observe upregulated expression of other inhibitory molecules, Lag3, 2B4 and CD160 in infected mice, and it will be interesting to investigate if these molecules also contribute to pathology and immune impairment during *C. trachomatis* infection.

While upregulation of the PD-1/PD-L1 pathway may be important in limiting damage to the host, there is no doubt that impairing the $CD8^+$ T cell response would also be beneficial to the invading pathogen. Although $CD4^+$ T cells are the main mechanism of *C. trachomatis* clearance during natural infection, it has been shown that *C. trachomatis* can be susceptible to the $CD8^+$ T cell response when these T cells are stimulated by vaccination or passively transferred (30). *C. trachomatis* may therefore benefit from mechanisms to subvert the $CD8^+$ T cells. Future experiments will be needed to determine whether *C. trachomatis* is capable of directly upregulating PD-L1 on infected cells. However, there is no evidence to suggest that PD-1/PD-L1 deficiency alone provides an optimal $CD8^+$ T cell response, and there may be other inhibitory mechanisms that contribute to the impaired $CD8^+$ T cell response during *C. trachomatis* infection, .

When T_{em} and T_{cm} cells were first described in the literature, data indicated that the T_{cm} cells were the more effective subset in terms of expansion and clearance capabilities (31). However, further studies demonstrated that the contribution of T_{cm} and T_{em} cells to pathogen control depends on the particular pathogen and the site of infection (32). For pathogens that replicate in lymphoid organs, T_{cm} cells are required for controlling pathogen burden. For example, T_{cm} cells are more efficient than Tem

memory $CD8^+$ T cells at lysing LCMV-infected cells (33). However, for mucosal infections, T_{cm} $CD8^+$ T cells are less effective since they are more confined to lymphoid organs and slow to respond to peripheral challenge. T_{em} cells, on the other hand, migrate through or reside in peripheral tissues and thus are closer to the site of the pathogen upon reinfection and can immediately engage the pathogen and limit its replication (34-36). The data presented here suggest that *C. trachomatis* impairs the $CD8^+$ T cell response by skewing it to a $T_{cm/n}$ phenotype. During the primary response to *C. trachomatis*, it is clear that PD-L1 expression results in an increased $T_{cm/n}$ $CD8^+$ T cell population while limiting the number of IFNγ producing $CD8^+$ T cells present in the uterus. Future experiments should compare the $T_{cm/n}$ populations in WT and PD-L1 deficient mice to determine if PD-L1 expression results in a higher proportion of naïve $CD8^+$ T cells. One possible explanation for the impaired $CD8^+$ T cell response may be that PD-L1 expression limits $CD8^+$ T cell priming and activation and results in a higher proportion of naïve $CD8^+$ T cells.

C. trachomatis is highly susceptible to IFNγ, and the increased percentage of IFNγ producing $CD8^+$ T cells, within the total T cell population may explain the enhanced clearance I observe during primary infection in PD-L1 deficient mice. However, how PD-L1 deficiency leads to an increased number of IFNγ $CD8^+$ T cells is still unclear. Evidence looking at the pro-apoptotic molecule, Bim, expression would suggest that PD-L1 leads to a disproportionate increase of Bim expression in T_{em} cells. This may be one mechanism to limit the expansion of T_{em} cells, thus decreasing the population that would readily produce IFNγ. Additionally, T_{em} cells are the population of $CD8^+$ T cells likely to be retained in the uterus, and this could explain the increased

population of *C. trachomatis* specific $CD8^+$ T cells in PD-L1 deficient mice following secondary infection. To test these hypotheses, future studies should perform detailed comparison of apoptotic markers, as well as markers of expansion, in T cells from PD-L1 deficient and WT mice.

Importantly these data show that memory cells generated in the absence of PD-1/PD-L1 signaling during *C. trachomatis* infection are able to provide superior protection compared to memory cells from WT mice. Although the major difference between T_{cm} and T_{em} cells may be in their capacity to migrate to peripheral tissues, our data indicate that there is a functional difference as well, and that this difference is mediated by the PD-1/PD-L1 interaction. Additionally these data provide insight into peripheral $CD8^+$ T cell responses in general. Unlike systemic infections, an effective secondary $CD8^+$ T cell responses in the periphery may be defined by the effector phenotype of the $CD8^+$ T cell population, and not by their capacity to greatly expand. Here I provide evidence that the PD-1/PD-L1 pathway is an important factor in regulating peripheral $CD8^+$ T cell responses by skewing the $CD8^+$ T cells to a more central memory phenotype, ultimately impairing the ability of these $CD8^+$ T cells to respond upon rechallenge. Little is known about the development of T_{cm} and T_{em} cells in the genital tract. I show that the T_{em} cell subset can be an important memory population in the genital tract during *C. trachomatis* infection. Mechanisms to promote the expansion and retention of effector memory $CD8^+$ T cells, whether by inhibiting PD-L1 or by other means, will be an important concept in the design of *Chlamydia* vaccines. An effective *C. trachomatis* vaccine may have to overcome inhibition mediated by PD-L1 to improve the $CD8^+$ T cell response without causing immunopathologies in the genital tract.

Materials and Methods

Mice

C57BL/6, B6.PL-Thy1a (CD90.1 congenic), and B6.129S7-IFNγtm1Agt (IFNγ-/-) mice were purchased from The Jackson Labs. PD-L1 and PD-1 deficient mice have been described previously and were generously provided by Arlene Sharpe. αPD-L1 blocking antibody (clone 10F.9G2) was generously provided by Gordon Freeman; αB7-1 blocking antibody (clone 1G10), IgG2a and IgG2b control antibodies were purchased from BioXCell. For transient inhibition experiments, mice were treated with 200μg αPD-L1 or αB7-1 each day for three days prior to infection and then every other day after infection. All animals were maintained and cared for within the Harvard Medical School Center for Animal Resources and Comparative Medicine (Boston, MA) (37, 38). All mice were treated with 2.5 mg medroxyprogesterone subcutaneously 7 days prior to infection with *C. trachomatis* to synchronize the murine estrous cycle. All experiments were approved by the Institutional Animal Care and Use Committee.

Growth, isolation, and detection of bacteria

C. trachomatis serovar L2 (434/Bu) was propagated within McCoy cell monolayers grown in Eagle's MEM (Invitrogen, Grand Island, NY) supplemented with 10% FCS, 1.5 g/l sodium bicarbonate, 0.1M nonessential amino acids, and 1 mM sodium pyruvate. Infected monolayers were disassociated from plates using sterile glass beads and were sonicated to disrupt the inclusion. Elementary bodies were purified by density gradient centrifugation, as described previously (39) . Aliquots were stored at -80°C in

medium containing 250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid and were thawed immediately prior to use. To quantify the levels of *C. trachomatis,* quantitative PCR (qPCR) with 16S primers specific for *C. trachomatis* was performed as has been previously described (39).

Flow cytometry

Tissues were mechanically disaggregated and immediately stained for surface markers or stimulated for 5 h with 50 ng/ml PMA (Alexis Biochemical) and 500 ng/ml ionomycin (Calbiochem) in the presence of brefeldin A (GolgiStop; BD Biosciences) for intracellular cytokine staining. Cells were preincubated with anti-FcRg (Bio X-Cell) before staining with αCrpA-APC (National Institute of Health Tetramer Core) or αPD-L1-APC, αCD4 Q-Dot, αCD8-APC-Cy7, and αCD90.2-PeCy7 (Biolegend). Cells were also incubated with αCD11b-PB, αCD11c-PB, αCD19-PB and αB220-PB to exclude these populations. For activation marker analysis, I examined αCD62L-FITC and αCD127-PerCP (BD Biosciences). Bim staining was performed with primary rabbit αBim followed by secondary αRabbit-PE (Cell Signaling). For intracellular cytokine staining αIFNγ PE (BD Biosciences) was used and cells were permeabilized with the Cytofix/Cytoperm Plus Kit according to the manufacturer's instructions (BD Biosciences). The absolute cell number in each sample was determined using AccuCheck Counting Beads (Invitrogen). Data were collected on an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star).

T cell depletion and transfer

For $CD8^+$ and $CD4^+$ T cell depletion experiments, mice were injected i.p. with 200 μg of anti-$CD8^+$ (clone 2.43), or 200 μg of anti-$CD4^+$ (clone GK1.5) or isotype control (clone LTF-2). Mice were treated with antibody every day starting 3 days prior to primary challenge and then every other day after challenge. Mice were sacrificed 5 days after challenge. For secondary challenge, mice were treated every day 3 days prior to secondary challenge and then every other day after secondary challenge.

For $CD8^+$ T cell transfer experiments, lymphoid organs and uteri were collected from naïve WT (Thy1.1) and PD-1 deficient (Thy1.1) mice. $CD8^+$ T cells were isolated using Dynal negative selection $CD8^+$ T cell kit (Invitrogen) and $5x10^6$ purified $CD8^+$ T cells were transferred i.v. into naïve Thy1.2 IFNγ deficient mice.

Generation of bone marrow chimeras

Bone marrow was obtained from femurs of WT and PD-L1 deficient mice. 10^7 WT and PD-L1 deficient bone marrow cells were injected i.v. into groups of PD-L1 deficient and WT mice irradated with 800 rads. Cells from these chimeras were analyzed after 6 weeks.

Statistical analysis

The data represent the mean ± SEM and were calculated using GraphPad Prism version 4.0. *P* values were determined using the nonparametric Mann-Whitney T test. Significant differences between groups are indicated as follows: *$p<0.05$, **$p< 0.01$, and ***$p<0.005$.

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Chapter Five: Discussion

The increase in *Chlamydia* infections over the last 20 years is a major concern for medical personnel, epidemiologists, researchers, and the millions of people infected. It has remained unclear what mechanisms prevent the development of sterilizing immunity during *C. trachomatis* infection. Without understanding the limitations of natural immunity, it will be difficult to design a vaccine that can circumvent or manipulate these limitations. Previous examination using the mouse model has demonstrated that both $CD4^+$ and $CD8^+$ T cells respond to infection, however an in depth analysis of the endogenous $CD8^+$ T cell response in the genital tract had not been performed. This thesis focused on understanding the kinetics of the endogenous $CD8^+$ T cell response to *C. trachomatis* and the mechanisms that act to limit that response.

***C. trachomatis* infection suppresses the mucosal $CD8^+$ T cell response.**

The upper reproductive tract requires a tightly regulated immune response. To overcome infection, the host must elicit protective immunity while limiting pathological responses that could cause permanent damage and negatively impact fertility. However, this goal is not achieved during infection with *C. trachomatis*. Rather, genital *C. trachomatis* infection results in severe immunopathology and very little protective immunity. Particularly interesting is that $CD8^+$ T cells derived from a natural *C. trachomatis* infection are not protective, while $CD8^+$ T cells derived outside a natural *C. trachomatis* infection are protective (1). Why are these potentially protective responses specifically impaired during *C. trachomatis* infection? This incongruence led me to more thoroughly investigate the natural $CD8^+$ T cell response during *C. trachomatis* infection of the genital tract.

In Chapter Two I showed that *C. trachomatis*-specific $CD8^+$ T cells responded upon primary infection, but that the secondary response was significantly impaired in both the dLNs and the genital tract. In comparison, transcervical infection with *L. monocytogenes* produced a much more robust secondary $CD8^+$ T cell response in the genital tract. It is unlikely that the limited secondary expansion in the dLNs contributed to the robust population observed in the genital tract during *L. monocytogenes* infection, as the numbers of $CD8^+$ T cells present in the genital tract were significantly greater than the $CD8^+$ T cells found in the dLNs at every time point (**Figures 2-9 and 2-10**). This evidence suggests that upon rechallenge in the genital tract, secondary $CD8^+$ T cell expansion may not occur in the dLNs. During *L. monocytogenes* secondary $CD8^+$ T cell expansion may occur in the genital tract. However, this hypothesis will be rigorously tested in the future by directly measuring and comparing $CD8^+$ T cell expansion in multiple tissues, including the dLNs, genital tract, spleen and liver during the course of *C. trachomatis* and *L. monocytogenes* infections. These studies will test the hypothesis that $CD8^+$ T cells are specifically impaired in their recall capacity during *C. trachomatis* infection by failing to expand or be recruited to the genital tract.

***C. trachomatis* alters the $CD8^+$ T cell response through the PD-1/PD-L1 pathway.**

In Chapter Four I describe how PD-L1 expression alters the $CD8^+$ T cell response to *C. trachomatis.* PD-L1 deficient mice that are infected with *C. trachomatis* exhibit lower primary bacterial burden compared to WT mice. I expected that this decrease in burden would correlate with an increase in the number of *C. trachomatis*-specific $CD8^+$ T cells present in the uterus during primary infection. I did not observe an increase in the

number of CrpA specific $CD8^+$ T cells, however I cannot rule out that other antigen-specific $CD8^+$ T cells may have increased in number (**Figure 4-6**). The most striking difference between WT and PD-L1 deficient mice, however, was the alteration in the phenotype of the $CD8^+$ T cells. PD-L1 deficient mice exhibited a significant increase in the ratio of effector memory (T_{em}) cells to naïve and central memory ($T_{cm/n}$) cells, and this correlated with an increase in the number of IFNγ producing $CD8^+$ T cells present in the genital tract. While depletion of $CD8^+$ T cells in PD-L1 deficient mice did not completely restore bacterial levels to that of WT, the altered $CD8^+$ T cells in PD-L1 deficient mice mediated the majority of protection observed (**Figure 4-10**).

I observed that PD-L1 deficiency restores the protective capacity of memory $CD8^+$ T cells. Memory $CD8^+$ T cells from PD-L1 deficient mice also exhibited an increase $T_{em}/T_{cm/n}$ ratio, and this likely resulted in an increase in the number of IFNγ-producing $CD8^+$ T cells that could quickly be activated upon secondary infection. An appropriate future direction for this work would be to test whether T_{em} cells are truly able to confer better protection compared to T_{cm} cells. To address this, one could transfer sorted T_{em} or T_{cm} cells from previously infected mice and compare *C. trachomatis* burden in infected recipient mice. It will be interesting to test not only which population confers protection in the absence of PD-L1/PD-1 signaling, but also to see if one population from WT mice is able to confer more or less protection. If T_{em} cells from WT mice are able to confer protection then this would provide support to the hypothesis that PD-L1 mediates its effects during *C. trachomatis* by limiting the $T_{em}/T_{cm/n}$ ratio. If T_{em} cells from WT are unable to confer protection, it would suggest that PD-L1 may act through a mechanism

other than or in addition to altering the $T_{em}/T_{cm/n}$ ratio in order to limit *C. trachomatis* clearance.

A final question that still remains is the mechanism by which PD-1/PD-L1 signaling alters the $T_{em}/T_{cm/n}$ ratio. When investigating the numbers of each population present in the uterus, it is clear that there is a slight increase in the number of T_{em} cells and a slight decrease in the number of $T_{cm/n}$ cells. However, there is no difference in the total number of memory cells present in the genital tract. These data suggest that PD-L1 does not limit the expansion of $CD8^+$ T cells, but alters the developmental pathway of $CD8^+$ T cells as they acquire memory phenotype. Since I did not distinguish between T_{cm} and T_n cells in my experiments, it is possible that the PD-1/PD-L1 pathway inhibited priming and activation of the $CD8^+$ T cells. To test this, it will be necessary to compare proportions of T_n and T_{cm} in WT and PD-L1 deficient mice. Alternatively it is possible that PD-L1 expression specifically drives $CD8^+$ T cells to become T_{cm}. Other studies have shown that PD-L1 expression limits the conversion of T_{cm} cells to T_{em}, however it is unclear how PD-L1 limits the conversion (2). In general $CD8^+$ T cells that experience enhanced TCR signals and longer duration of signal develop into T_{em} cells that do not progress to T_{cm} cells or take a longer amount of time to progress to T_{cm} cells (3). This would indicate that the T_{em}/T_{cm} ratio is established during priming, and would concur with my data indicating that PD-L1 blockade during priming alters the $CD8^+$ T cell response during secondary infection (**Figure 4-6**). By inhibiting downstream TCR signaling in $CD8^+$ T cells, it is likely that the PD-L1/PD-1 signaling drives the $CD8^+$ T cell down a T_{cm} developmental pathway. Alternatively, it may be that PD-L1 signaling does not necessarily promote conversion of T_{em} cells to T_{cm} cells but actually results in

increased apoptosis of T_{em} cells specifically. In support of this hypothesis I have found that expression of the pro-apoptotic gene Bim is significantly lower in T_{em} cells of PD-L1 deficient mice compared to WT mice. Future studies should investigate the expression of other apoptosis markers to better understand if PD-1/PD-L1 signaling differentially affects apoptotic potential in T_{cm} and T_{em} cells.

In the interest of developing therapeutics, future studies should also investigate how the timing of PD-L1 expression alters the T_{em}/T_{cm} ratio. If the T_{em}/T_{cm} ratio is established during priming as I hypothesize, is it possible to alter the ratio after the memory $CD8^+$ T cell population has been established? To investigate this question, one should treat previously infected mice with anti-PD-L1 antibody. Memory cells should be collected and transferred to congenic naïve mice; after recipient mice are infected the T_{em}/T_{cm} ratio should be measured in the transferred memory $CD8^+$ T cells. This approach will allow researchers to test how anti-PD-L1 treatment specifically affects already established memory T cell populations. If anti-PD-L1 treatment alters the T_{em}/T_{cm} ratio after memory formation then anti-PD-L1 antibody might be a valuable treatment option to improve the immune response in *C. trachomatis*-infected patients.

Model for how PD-L1 limits *C. trachomatis* clearance

The section above describes how the PD-1/PD-L1 pathway drives the $CD8^+$ T cell response towards a $T_{cm/n}$ phenotype. The data presented in this thesis provide an interesting model that could explain how PD-L1 deficiency alters the $CD8^+$ T cell response to improve bacterial clearance. During primary infection, PD-L1 expression on epithelial cells may directly protect these cells from cytolytic killing by $CD8^+$ T cells by

disengaging the $CD8^+$ T cell. Alternatively, when $CD8^+$ T cells are engaged by PD-L1 it may cause transcriptional changes in the $CD8^+$ T cell that lead to the $T_{cm/n}$ phenotype and a dampening of the cytolytic response. The interaction with PD-L1 expressing epithelial cells leads to inefficient bacterial clearance during primary infection, which can be reversed by PD-L1 deficiency or blockade. In the absence of PD-L1, $CD8^+$ T cells have an enhanced ability to recognize and engage infected cells or have an improved cytolytic capacity that contributes to bacterial clearance. Future experiments should test the cytolytic response of $CD8^+$ T cells to infected cells *in vitro* with and without PD-L1 blockade to investigate if $CD8^+$ T cell cytokine production is reduced in the presence of PD-L1.

Once engaged by PD-L1, the $CD8^+$ T cell may be headed down a transcriptional course that results in the increased expression of PD-1 and the development of the $T_{cm/n}$ phenotype. PD-1 expression indicates that $CD8^+$ T cells may be exhausted as infection is cleared and memory is established, thus T cells are more prone to apoptosis and have a decreased ability to produce cytokines. Furthermore, the PD-L1/PD-1 interaction drives $CD8^+$ T cells to a $T_{cm/n}$ phenotype, which further disables the secondary response by sequestering antigen-experienced $CD8^+$ T cells from the genital tract. Upon reinfection the secondary $CD8^+$ T cells that are able to migrate to the genital tract have lost the capacity to produce IFNγ and other inflammatory cytokines, thus these $CD8^+$ T cells do not contribute to secondary bacterial control. In the absence of PD-L1, adaptive immune responses other than $CD8^+$ T cells are able to quickly control infection. However, in the absence of PD-L1, $CD8^+$ T cells become sufficient for controlling infection. Future studies should investigate the transcriptional changes that occur in $CD8^+$ T cells

following *C. trachomatis* infection in the presence and absence of PD-L1. It will be interesting to test if the transcriptional profile of memory $CD8^+$ T cells from *C. trachomatis* is similar to what has been described for exhausted $CD8^+$ T cells in other models (4).

Effector memory and Central memory T cell responses in the genital tract

Mucosal surfaces require $CD8^+$ T cells that can easily and quickly access infected epithelial cells. T_{em} cells are specialized for mucosal responses. T_{em} cells shed surface expression of the lymph node homing molecule, CD62L, after activation and can therefore reside at length in mucosal tissues and be activated by infected epithelial cells. In contrast, T_{cm} and naïve cells maintain high levels of CD62L, migrate through the vasculature, and require pAPCs to become activated. During transcervical infection with *L. monocytogenes,* I observed a higher ratio of T_{em} to $T_{cm/n}$ cells present in the genital tract compared to infection with *C. trachomatis*. Yet, I still observed a significant $T_{cm/n}$ population present in the genital tract, indicating that these cells may be circulating through the genital tract as well. The presence of $T_{cm/n}$ cells also indicates that T_{em} cells must compete with this population for antigen. A high T_{em} to $T_{cm/n}$ ratio would favor expansion of the T_{em} population. Thus the increased presence of T_{em} cells in the genital tract, which can be activated by infected epithelial cells, likely contributes to the robust secondary response observed with *L. monocytogenes* infection. During *C. trachomatis* infection, however, the low T_{em} to $T_{cm/n}$ ratio indicates that T_{em} cells are outcompeted for antigen by T_{cm} cells. T_{cm} cells require pAPCs for activation and subsequent expansion. However, I've shown that the DC population in the uterus is much lower than that of the

epithelial population, and additional evidence from our lab has shown that the DC population may be functionally impaired during *C. trachomatis* infection (X. Zhang, unpublished). Therefore, the T_{cm} population would have an abrogated ability to become activated and expand upon secondary infection. Ultimately, a detailed comparison of the T_{em} and T_{cm} responses in the genital tract during primary and secondary *C. trachomatis* infections will need to be performed using proliferation makers to fully understand these differences in expansion.

The different cytotoxic capacities of T_{em} and $T_{cm/n}$ cells predict that these cells differentially contribute to protection against viral, bacterial and host driven assaults. Transcervical immunization with *L.m.*-CrpA, which resulted in a high $T_{em}/T_{cm/n}$ ratio, protected mice against subsequent *C. trachomatis* infection. It is likely that the enhanced capacity of T_{em} cells to produce IFNγ contributed to protection against *C. trachomatis* infection. Interestingly, immunization with *L.m.*-Ova, which shares no known antigen with *C. trachomatis,* also resulted in a level of protection against *C. trachomatis* infection. These results suggest that T_{em} cells have a more potent bystander effect, meaning they can be non-specifically activated, compared to $T_{cm/n}$ cells that contributes to *C. trachomatis* clearance. In order to test this hypothesis in the future, it will be necessary to deplete mice of $CD8^+$ T cells after *L.m.*-OVA infection to determine if this population indeed contributes bystander protection against *C. trachomatis.*

Immuno-inhibitory pathways involved in the immune response to *C. trachomatis*

C. trachomatis is a pathogen that is highly adapted to live within its human host. As such, *C. trachomatis* has evolved mechanisms to inhibit host immunity to further its persistence and enhance its transmission. The work in this dissertation describes one mechanism that *C. trachomatis* uses to limit the $CD8^+$ T cell response and enhance its own survival in the host. I hypothesized that *C. trachomatis*, like other chronic pathogens, may cause the upregulation of immuno-inhibitory pathways to suppress the host immune response. I found that in fact several immuno-inhibitory pathways are upregulated during transcervical infection with *C. trachomatis*. My data focused on the biological consequences of PD-L1 expression, however I also observed an upregulation of the inhibitory molecules Lag3, CD160 and 2B4 was also significantly upregulated during *C. trachomatis* infection.

Lag3 is expressed on a variety of lymphocytes, including NK cells, B cells and T cells (5, 6). Lag3 expressed by tolerized T cells is thought to have a role in limiting T cell responses to self-antigen (7). However, Lag3 expression following infection can also indiscriminately limit pathogenic responses by T cells. Expression of Lag3 on regulatory T cells (Tregs) causes these cells to directly suppress effector functions of other T cells (7). In my studies I did not explore the role of Lag3 or Tregs in regulating immunity towards *C. trachomatis*, however it is reasonable to hypothesize that Lag3 expression could contribute to the suppression of $CD8^+$ T cell responses during *C. trachomatis* infection. Interestingly CD160 has dual roles of stimulation and inhibition of T cell responses (8). CD160 binding to MHC I enhances cytolytic activity and cytokine production of $CD8^+$ T cells (9). However, CD160 binding to the Herpesvirus Entry Mediator (HVEM) on $CD4^+$ T cells inhibits $CD4^+$ T cell activation (10). It will be

interesting to investigate if CD160 plays a role in the $CD8^+$ and/or the $CD4^+$ T cell responses to *C. trachomatis*. The expression of 2B4 also exhibits dual functions. The expression of 2B4 on $CD8^+$ T cells during chronic viral infections limits secondary T cell expansion (11). However, 2B4 expressing natural killer (NK) cells engage CD48 on target cells which leads to enhanced cytotoxicity and IFNγ production (12).

Ultimately there may be several mechanisms that contribute to suppressing the immune response against *C. trachomatis*. Other studies have found that immuno-inhibitory molecules such as the PD-1 and Lag3 act cooperatively to inhibit $CD8^+$ T cell responses, and combinatorial blockade of these molecules further enhances $CD8^+$ T cell mediated immunity compared to either single blockade (13, 14). Future studies should not only examine the biological roles of each of these molecules alone during *C. trachomatis* infection, but also explore how combinations of these molecules might synergistically act to alter the immune response to *C. trachomatis*.

Host mediated versus *C. trachomatis* mediated immune-inhibition

The question remains whether the upregulation of these immuno-inhibitory pathways is mediated directly by *C. trachomatis* or mediated by the host to limit damaging pathology. It is possible that *C. trachomatis* expresses a factor, e.g. type three secreted effector, which directly alters expression of one or more of these immuno-inhibitory pathways. Although direct manipulation of immuno-inhibitory molecules by a pathogen has not been yet described, alteration of host transcription by pathogens is well documented (15-17). Since *C. trachomatis* has a type III secretion system (T3SS), any one of these secreted effectors or proteins present in the inclusion membrane, would have

access to the host cytosol and could potentially alter expression of a number of genes, including immuno-inhibitory genes. To investigate whether a *C. trachomatis* T3SS effector can directly modulate the expression of an immuno-inhibitory molecule, one should singly express the secreted effectors of *C. trachomatis* in tissue culture cells and observe if any one of these can recapitulate the increased expression of PD-L1 and/or other immuno-inhibitory molecules.

In order to understand if these immuno-inhibitory pathways contribute to limiting pathology of the host, a thorough histopathology analysis will need to be performed in mice that are either deficient in these molecules or treated with blocking antibodies. Although my own studies did not uncover consistent differences in pathology between WT and PD-L1 deficient mice, it's possible that multiple pathways are involved with limiting inflammation and subsequent pathology. Furthermore, it would be interesting to investigate if there is a connection between expression of immuno-inhibitory molecules within the genital tracts of patients that have or have had *C. trachomatis* infections and either fully recover or develop inflammatory sequelae such as endometritis or pelvic inflammatory disease (PID). Currently the reported rate at which women develop PID following *C. trachomatis* infection ranges from 3% to 30% (18). It will be useful for clinicians to understand if immuno-inhibitory molecules contribute to the risk of developing an inflammatory condition.

Identification of the mechanisms that alter both $CD4^+$ and $CD8^+$ T cell responses will allow researchers to manipulate these mechanisms and ultimately contribute to more effective treatment and vaccine design. Combining antibiotic treatment with blockade of immuno-inhibitory molecules might prove to be a promising therapeutic that could

enhance the host's natural immune response against *C. trachomatis* infection. However the delicate balance between enhancing the immune response and limiting damaging pathology should obviously be considered.

Other *C. trachomatis* mediated diseases

Importantly it will be of interest to know if PD-L1 expression also limits *C. trachomatis* clearance and promotes disease in other tissues. In addition to the genital mucosa, *C. trachomatis* is a pathogen of the ocular tissue. PD-L1 expression on retinal epithelial cells has been shown to inhibit cytotoxic function of $CD8^+$ T cells to maintain an immunosuppressive environment (19). As an immune privileged site, it will be important to understand if the role that PD-L1 plays in the genital mucosal also extends to the ocular mucosa during *C. trachomatis* infection.

Interestingly, *C. trachomatis* is the most common bacterial trigger of reactive arthritis (ReA) (20). *C. trachomatis* induced ReA occurs when *C. trachomatis* infected macrophages or monocytes migrate into the synovial tissue (21). *C. trachomatis* can enter into a persistent, non-replicative, state in which immune cells and IFNγ become much less effective, thus allowing *C. trachomatis* to persist in the tissue. Occasionally patients with ReA exhibit acute inflammation, and this is probably due to *C. trachomatis* transitioning from persistence to a metabolically active form. While the exact immune mechanisms that mediate *C. trachomatis* ReA are unclear, patients who exhibit chronic ReA also exhibit much lower levels of IFNγ compared to patients that resolve ReA (22). Although counterintuitive, these data suggest that an increase in the cellular populations that secrete inflammatory IFNγ may help resolve ReA. PD-L1 deficiency contributes to

T cell hyperactivity, which would promote the inflammation characteristic of arthritis, however in the context of *C. trachomatis*-induced ReA effector T cells may be necessary to resolve infection. It will be interesting to investigate whether PD-L1 plays a role in limiting or promoting the symptoms of ReA.

C. trachomatis suppresses the $CD8^+$ T cell response to other pathogens

C. *trachomatis* infections are highly associated with the occurrence of other sexually transmitted infections (STI). There are obvious lifestyle factors that contribute to this association. In addition, the evidence I present in Chapter Two indicates that infection with *C. trachomatis* may suppress the immune response to other pathogens. By broadly suppressing the $CD8^+$ T cell response, *C. trachomatis* may limit the host's ability to clear other infections of the genital tract. The data presented in Chapter Two demonstrate that prior *C. trachomatis* infection reduced the number of activated $CD8^+$ T cells in the uterus following infection with *L. monocytogenes*. Prior infection with *C. trachomatis* skewed the $T_{em}/T_{cm/n}$ ratio towards more $T_{cm/n}$ cells, and this could not be overcome by subsequent infection with *L. monocytogenes.* It is the shift reducing the $T_{em}/T_{cm/n}$ ratio that may contribute to suppressing the $CD8^+$ T cell response in the genital tract. Whether *C trachomatis* infection is altering the phenotype of *L. monocytogenes*-specific $CD8^+$ T cells in the genital tract is unclear. However, if the data from the dLNs, showing that co-infection with *C. trachomatis* results is a significant decrease in the number of IFNγ producing *L. monocytogenes*-specific $CD8^+$ T cells, is an indication then it is likely that *C. trachomatis* infection does alter the *L. monocytogenes* specific $CD8^+$ T cell response in the genital tract. In order to test this hypothesis, tetramer staining of a

dominant *L. monocytogenes* antigen-specific $CD8^+$ T cell population would have to be performed with and without *C. trachomatis* co-infection. Furthermore, these *C. trachomatis*-induced T cell changes did not affect clearance of *L. monocytogenes* at the particular time point tested, although future studies should investigate if *L. monocytogenes* exhibits delayed clearance at later time points.

C. trachomatis infection must occur prior to infection with *L. monocytogenes* to alter the $CD8^+$ T cell response, no effect is observed when coinfection occurs simultaneously. This suggests that the mechanism, which alters the $CD8^+$ T cell response, is occurring extremely early during *C. trachomatis* infection that ultimately affects the general priming of $CD8^+$ T cells. Based on the evidence presented in Chapter Three, the upregulation of an inhibitory pathway may contribute to this suppression, although future studies will confirm this. What will be interesting for future investigations is whether prior *C. trachomatis* infection, that has been cleared, also suppresses the $CD8^+$ T cell response to a subsequent infection by a different pathogen. If $CD8^+$ T cell suppression is long term, this would suggest that people who have had previous *C. trachomatis* infections have an increased risk for contracting another STI. Furthermore, it will be necessary to understand if genital infection with *C. trachomatis* is able to suppress the $CD8^+$ T cell response in other mucosal organs.

Developing a vaccine against *C. trachomatis*

As the most highly reported communicable disease in the United States, developing an effective vaccine to limit contraction and spread of *C. trachomatis* is of vital importance. Previous efforts for designing a *C. trachomatis* vaccine have focused

on identifying protective antibody responses. In particular, researchers have focused on antibody responses to the major outer membrane protein (MOMP) of *C. trachomatis.* The data examining the protective effects of immunization against MOMP have revealed varying results. Several studies have shown that it is possible to immunize against MOMP, using various vehicles, to elicit partial protection to *C. trachomatis* challenge (23, 24). However, other studies have indicated that immunization against MOMP produces limited or no measurable protection compared to non-immunized animals (25, 26). Additionally, it has proved difficult to elicit a pan-serovar response that is protective against the multiple serovars of *C. trachomatis.* The discrepancies in the many *Chlamydia* vaccine studies may arise due to the route of immunization, different adjuvants used, and strain of *Chlamydia* studied. Future work should aim to optimize the vaccine delivery and adjuvants used such that cross-strain protection is achieved.

While studying protective antibody responses should prove fruitful, researchers should also investigate protective T cell responses that could be apart of a multisubunit vaccine. Because $CD8^+$ T cells are able to identify and lyse infected cells, this population should be an integral part of the immune response to *C. trachomatis.* Furthermore, the work in this thesis and that of others has shown that it is possible to generate a protective $CD8^+$ T cell response against *C. trachomatis* (1). To date there has not been an effective T cell vaccine against a microbial pathogen. However, advances in our knowledge of the heterogeneity of $CD8^+$ T memory cells have informed researchers as to the particular $CD8^+$ T cell types that can be protective against different challenges, and the ways to manipulate specific responses. This has led to recent clinical trials testing the efficacy of a $CD8^+$ T cell vaccines against different types of cancers (27-30). Furthermore,

researchers are now studying how to improve $CD4^+$ and $CD8^+$ T cell responses against HIV through vaccination (31). The work in this thesis has provided the hypothesis that generating a T_{em} specific response against *C. trachomatis* will provide long-term immunity in the genital tract. Future work should aim to understand if T_{em} cells have superior protection against *C. trachomatis* as well as other genital pathogens, such as HIV and *Gonorrhea neisseria.* Furthermore, it will be important to investigate how to elicit protective T cell responses in the genital tract through vaccination in combination with different adjuvants or blockade of inhibitory pathways.

The work of this thesis demonstrated that the PD-1/PD-L1 pathway alters the $T_{em}/T_{cm/n}$ ratio, which may ultimately lead to less protection against *C. trachomatis*. The PD-1/PD-L1 pathway negatively regulates the immune response to many viral pathogens and in many cancer models. There is great interest in understanding how to manipulate the PD-1/PD-L1 pathway to improve the immune response in combination with vaccination, particularly in cancer models. Evidence in mice shows that antibody blockade of PD-1 improves effector T cell responses and inhibits tumor cell dissemination (32-34). Furthermore, several recent clinical trials have demonstrated that patients treated with anti-PD-1 or anti-PD-L1 antibody have slower tumor progression (35-37). These trials show the promising potential of PD-1/PD-L1 blockade as an anti-cancer therapy, and provide support that PD-1/PD-L1 blockade may be a beneficial treatment for other diseases as well.

Concluding remarks

The immune response to a chronic infection such as *C. trachomatis* involves the delicate balance of limiting host pathology while mounting an effector response capable of restricting bacterial replication. By impairing elements of the adaptive immune response, *C. trachomatis* is able to tip the balance to favor its own replication. This dissertation has identified the PD-1/PD-L1 pathway as one mechanism that inhibits the $CD8^+$ T cell response to *C. trachomatis* and prevents efficient clearance. The findings presented here, and future studies aimed at understanding the role of other immuno-inhibitory molecules in altering adaptive immunity, will aid the development of potential therapeutics and vaccines against *C. trachomatis.*

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Appendix A: Characterizing the $CD8^+$ T cell response to the *C. trachomatis* antigen PmpI

The data presented in Chapter 2 thoroughly describes the $CD8^+$ T cell response to the *C. trachomatis* antigen CrpA. I wanted to understand if the response observed for CrpA was specific to that antigen or representative of the $CD8^+$ T cell response to other *C. trachomatis* antigens. At the time of these studies the only other known $CD8^+$ T cell antigen identified in C57/BL6 mice was Polymorphic membrane protein I (PmpI) (1). PmpI is part of a family of proteins found in the outer membrane of *C. trachomatis.* I decided to first compare the systemic responses to CrpA and PmpI to ensure that I could appropriately monitor the PmpI specific response. I intravenously infected mice with *C. trachomatis* and measured the CrpA and PmpI specific responses by staining with tetramer specific for each antigen. The number of PmpI specific $CD8^+$ T cells in the spleen was about 10 fold lower than the number of CrpA specific $CD8^+$ T cells (**Figure A-1**).

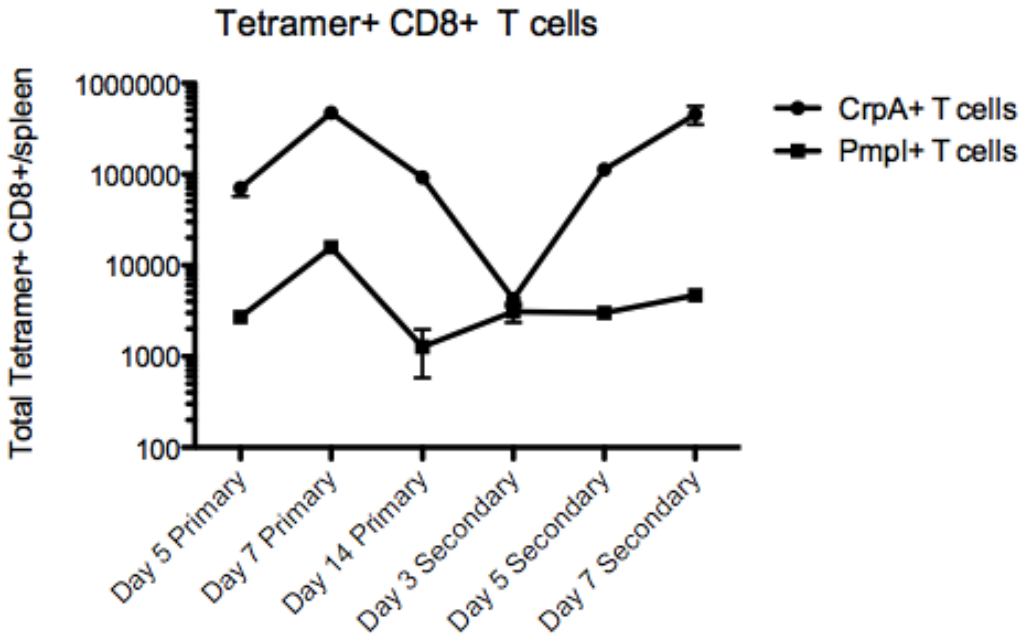

Figure A-1 Comparison of the PmpI and CrpA specific CD8$^+$ T cell responses during systemic infection. Mice were intravenously infected with 10^7 IFU of *C. trachomatis*. At indicated time points during primary and secondary infection spleens were harvested and tetramer$^+$ CD8$^+$ T cells were measured by flow cytometry. Each time point is the mean of 5 mice per group and error bars represent SEM.

Although the number of PmpI specific CD8$^+$ T cells was low compared to the number of CrpA specific CD8$^+$ T cells during systemic infection I decided to investigate the PmpI specific response during transcervical infection. I infected mice with 10^6 IFU of *C. trachomatis*; seven days following infection I measured the number of PmpI and CrpA specific CD8$^+$ T cells in the genital tract by flow cytometry. I was unable to detect any significant difference in the PmpI population in infected mice compared to uninfected mice (**Figure A-2**).

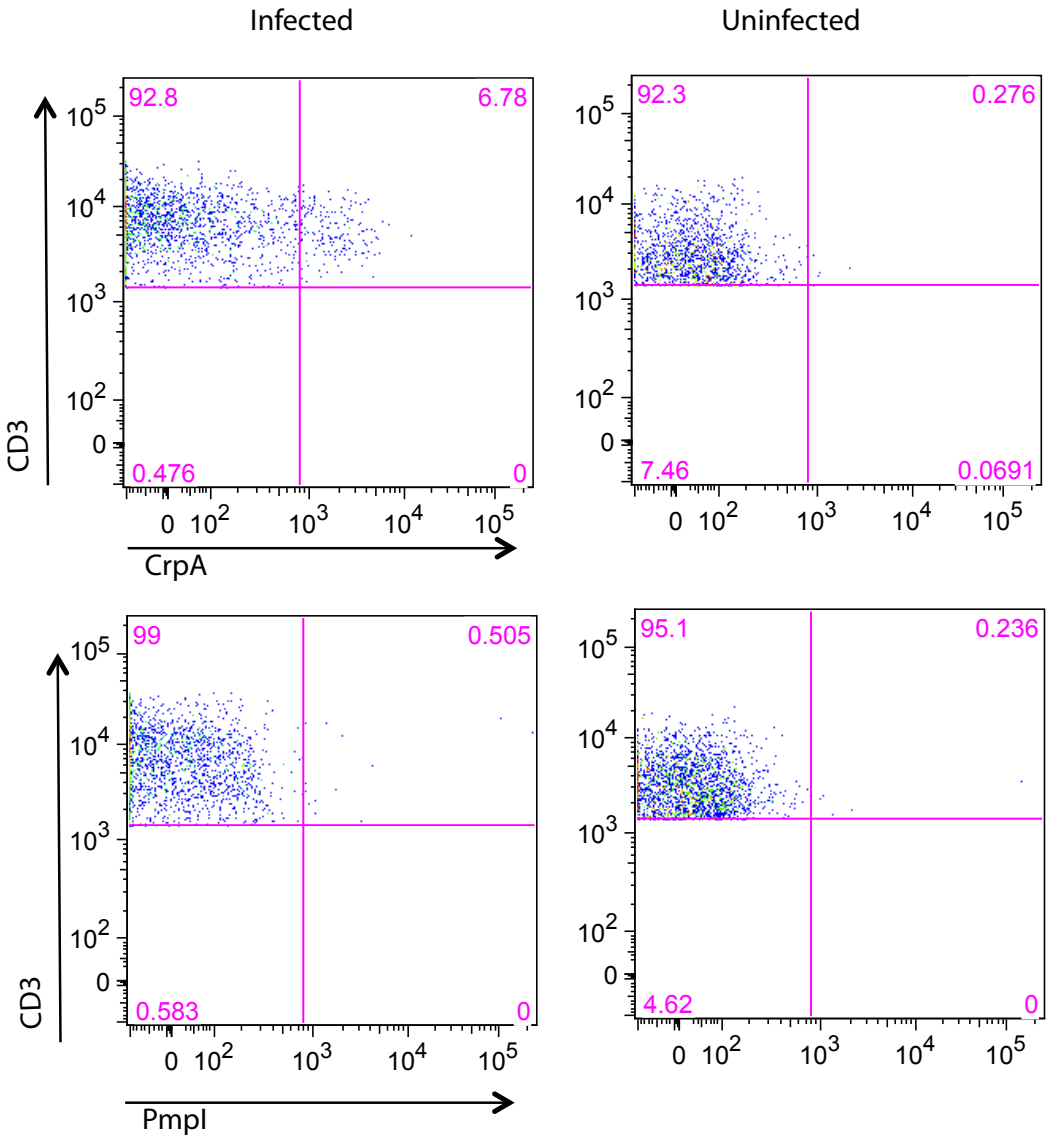

Figure A-2 PmpI specific CD8$^+$ T cells are undetectable in the genital tract during transcervical *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis.* Seven days post infection genital tracts were harvested and tetramer$^+$ cells measured by flow cytometry. Shown are representative flow plots of infected and uninfected mice for CrpA tetramer (top) and PmpI tetramer (bottom). Cells were first gated on CD90.2$^+$, CD4$^-$, CD8$^+$ lymphocyte populations. Tetramer$^+$ CD8$^+$ T cells are present in the top right quadrant of each plot.

Since I was unable to detect PmpI specific $CD8^+$ T cells in the uterus, I questioned whether this was due to a low abundance of PmpI specific $CD8^+$ T cells or possibly an issue with the tetramer. To test this, I transcervically infected mice with *C. trachomatis* and measured the PmpI specific $CD8^+$ T cells present in the dLNs by ELISPOT assay over a time course of infection. I was able to detect an expansion of PmpI-specific $CD8^+$ T cells in dLNs during primary infection; with the peak of the response occurring five days post infection (**Figure A-3**). By seven days post infection, the number of PmpI specific $CD8^+$ T cells had returned to levels similar to mice at day zero. After mice were rechallenged with *C. trachomatis* I observed a slight increase in the number of PmpI specific $CD8^+$ T cells, however the peak of secondary response was significantly lower than the peak of primary (**Figure A-3**). All together these data demonstrate that PmpI specific $CD8^+$ T cells appear to be at a lower abundance compared to CrpA specific $CD8^+$ T cells. By ELISPOT, at each time point the number of PmpI specific $CD8^+$ T cells detected was about 10 fold less than the number of CrpA specific $CD8^+$ T cells detected by ELISPOT (**Figure 2-1)**. However, the PmpI response was still similar to the CrpA response in that the secondary response was significantly lower than the primary response. Thus these data indicate that the CrpA specific $CD8^+$ T cell response characterized in Chapter Two is representative of other *C. trachomatis* antigens.

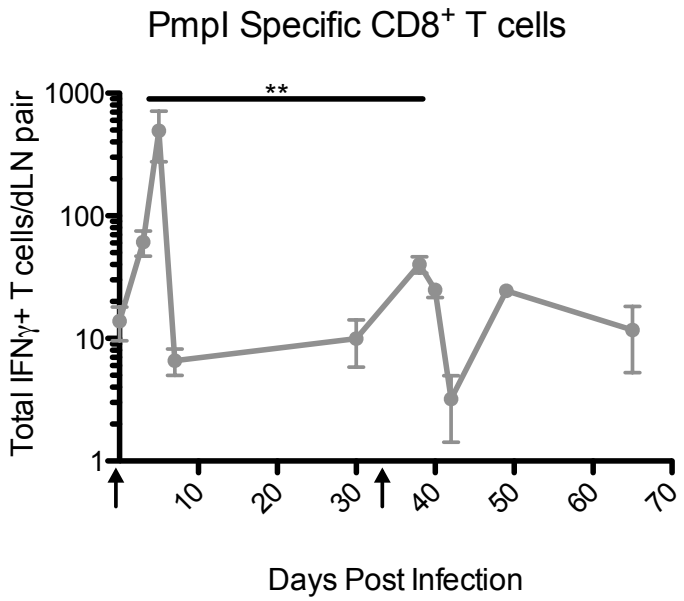

Figure A-3 PmpI specific CD8$^+$ T cells are stimulated during transcervical *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis* on day 0 (marked by 1st arrow). At timepoints marked, dLNs were harvested and the number of PmpI specific IFNγ producing CD8$^+$ T cells were measured by ELISPOT. Mice were rechallenged at the second arrow. Each timepoint is the mean of five mice per group, and bars represent SEM. Statistical significance of **$p<0.01$ is indicated for day 5 primary and day 5 secondary infections. Statistics were determined by Mann-Whitney Test.

The low abundance of PmpI specific CD8$^+$ T cells led me to hypothesize that PmpI is expressed at extremely low levels during infection, or is unable to access the host cytosol, and thus is unavailable to be presented on MHC class I machinery and stimulate a CD8$^+$ T cell response. However, transcriptional evidence from the Caldwell lab suggested that PmpI is actually expressed at higher levels compared to CrpA, and both PmpI and CrpA are expressed during the mid to late cycle of infection (2). With this information I hypothesized that PmpI is actually a weaker antigen compared to CrpA. To test these hypotheses I engineered a *L. monocytogenes* strain expressing PmpI fused to

the Ova SIINFEKL sequence (*L.m.*-PmpI). If PmpI expression or access to the host cytosol are limiting factors in stimulating a robust $CD8^+$ T cell response during *C. trachomatis* infection, then infection with *L.m.*-PmpI should be able to produce a $CD8^+$ T cell response that is comparable to *L.m.*-CrpA. If, however, PmpI is truly a weaker antigen compared to CrpA, then the $CD8^+$ T cell response to PmpI should be low regardless of the vector delivering the PmpI antigen. I intravenously infected mice with either *L.m.*-CrpA or *L.m.*-PmpI. Seven days post infection I measured the number of CrpA and PmpI specific $CD8^+$ T cells. Because CrpA and PmpI were both fused to the Ova antigen I could also measure the number of Ova specific $CD8^+$ T cells using an Ova specific pentamer. Infection with *L.m.*-PmpI produced significantly fewer PmpI-specific $CD8^+$ T cells compared to the number of CrpA specific $CD8^+$ T cells produced by *L.m.*-CrpA (**Figure A-4**). However, both *L.m.*-PmpI and *L.m.*-CrpA produced similar percentages of Ova specific $CD8^+$ T cells (**Figure A-4**). These data confirm that the low percentage of PmpI specific $CD8^+$ T cells is not due to limited access to the cytosol or low expression levels during *C. trachomatis* infection.

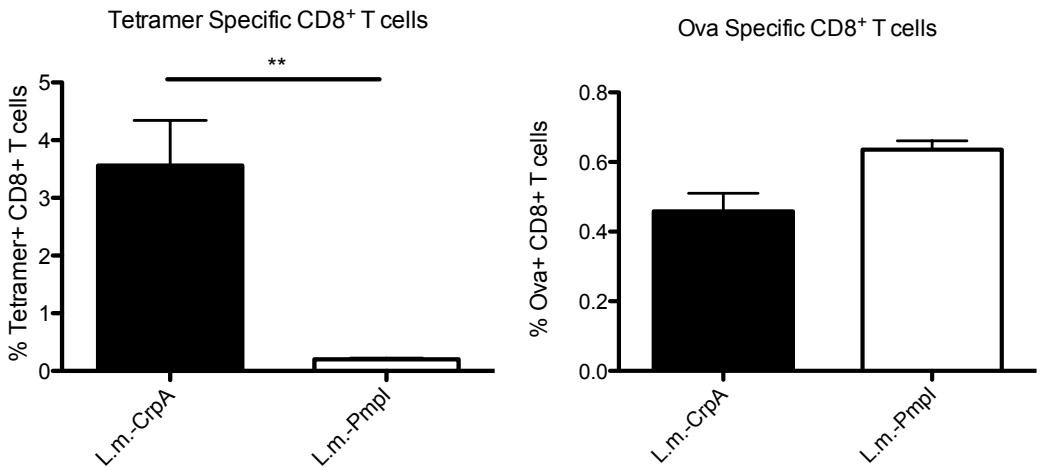

Figure A-4. Infection with *L.m.*-PmpI produces few PmpI CD8$^+$ T cells. Mice were intravenously infected with 10^5 CFU of *Lm*-CrpA or *L.m.*-PmpI. Seven days post infection spleens were harvested and tetramer specific CD8$^+$ T cells measured by flow cytometry. Spleenocytes were stained with CrpA or PmpI tetramer (left graph) or Ova Pentamer (right graph). Shown is the mean of 5 mice per group and error bars represent SEM. Statistical significance is indicated by **$p<0.01$, determined by the Mann-Whitney Test.

The data presented above provides compelling evidence that PmpI is in fact a weaker antigen compared to CrpA. However, I had yet to rule out the possibility that the differences in CD8$^+$ T cell abundance were due to tetramer staining. To test this possibility, I transcervically infected mice with either *L.m.*-CrpA or *L.m.*-PmpI. Seven days post infection I measured the number of CrpA , PmpI, and Ova specific IFNγ producing CD8$^+$ T cells by ELISPOT assay. Similar to the tetramer data, infection with *L.m.*-PmpI produced far fewer PmpI specific CD8$^+$ T cells compared to the CrpA specific response elicited by *L.m.*-CrpA (**Figure A-5**). *L.m.*-PmpI and *L.m.*-CrpA produced similar numbers of Ova specific CD8$^+$ T cells (**Figure A-5**). These data confirm that PmpI is a weaker antigen than CrpA. Additionally these data suggest that the low

antigenicity of PmpI would make it difficult to study the endogenous PmpI response during transcervical infection.

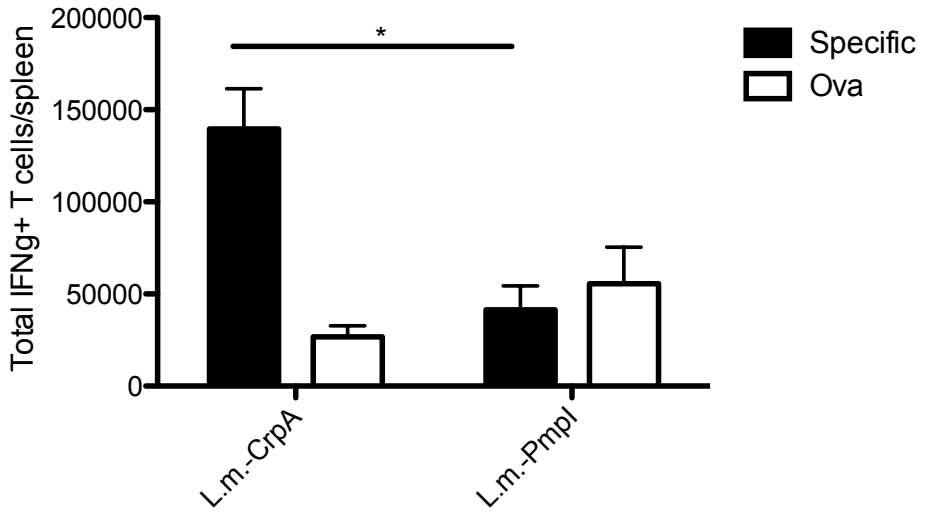

Figure A-5 PmpI elicits a weaker CD8$^+$ T cell response compared to CrpA. Mice were intravenously infected with 10^5 CFU of *Lm*-CrpA or *L.m.*-PmpI. Seven days post infection spleens were harvested and CrpA, PmpI and Ova specific IFNγ-producing CD8$^+$ T cells measured by ELISPOT assay. Black bars represent CrpA and PmpI specific responses to *L.m*-CrpA and *L.m.*-PmpI infections respectively. White bars represent the Ova specific response. All bars represent the mean of 5 mice per group and error bars represent SEM. Statistical significance is indicated by *$p<0.05$, determined by the Mann-Whitney Test.

Although PmpI is a weaker antigen compared to CrpA, I was curious if this antigen could still confer protection against *C. trachomatis* infection. To test this, I intravenously immunized mice with *L.m.* strains expressing CrpA, PmpI or Ova, or immunized mice with *C. trachomatis.* Twenty-eight days after immunization I challenged mice with 10^6 IFU of *C. trachomatis.* Three days post infection I measured *C. trachomatis* burden in the spleen. Mice immunized with *L.m.*-CrpA showed bacterial

levels equivalent to mice immunized with *C. trachomatis,* and significantly lower *C. trachomatis* levels compared to unimmunized mice and mice immunized with *L.m.*-Ova (**Figure A-6**). Mice immunized with *L.m.*-PmpI showed *C. trachomatis* levels comparable to unimmunized mice. These data confirm that mice immunized against PmpI are not protected against subsequent challenge with *C. trachomatis.*

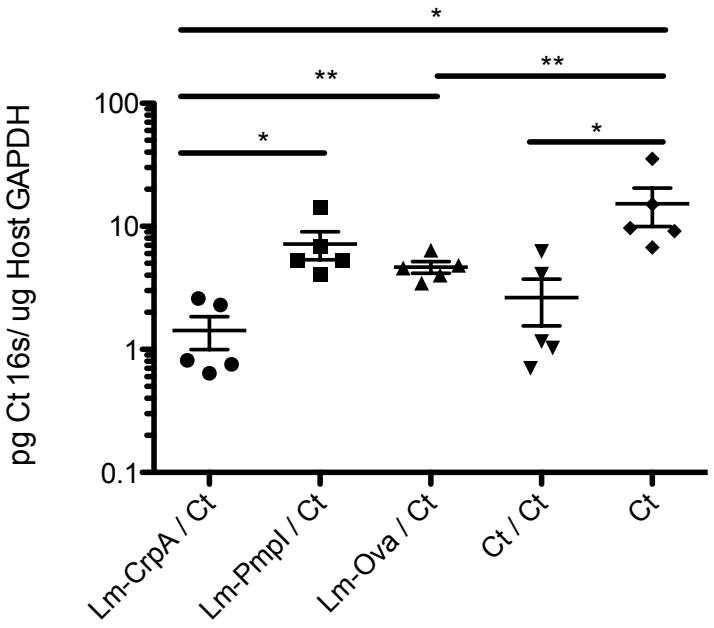

Figure A-6 Immunization with *L.m.*CrpA, but not *L.m.*-PmpI, protects against *C. trachomatis* challenge. Mice were intravenously immunized with 10^5 CFU of *Lm*-CrpA , *L.m.*-PmpI, *L.m.*-Ova, or 10^7 IFU of *C. trachomatis,* or left unimmunized. Twenty-eight days post infection mice were rechallenged intravenously with 10^7 IFU of *C. trachomatis.* Three days after rechallenge bacterial levels in the spleen were measured by qPCR. in spleens. Shown is the mean of 5 mice per group and error bars represent SEM. Statistical significance is indicated by: *$p<0.05$ and **$p<0.01$, determined by the Mann-Whitney Test.

All together these data indicate that PmpI elicits a poor $CD8^+$ T cell response that is not protective against *C. trachomatis.* Furthermore, the paucity of the $CD8^+$ T cell

response suggests that it is not a suitable antigen for further study during transcervical *C. trachomatis* infection. Since these studies were conducted, four more *C. trachomatis* $CD8^+$ T cell antigens have been identified (3). It will be interesting to investigate the $CD8^+$ T cell responses to these antigens and to understand if immunizations against such antigens produce protective $CD8^+$ T cell responses similar to CrpA.

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Appendix B: Supplemental data of Chapter Four

PD-1 deficiency recapitulates the phenotype of PD-L1 deficiency

As described in Chapter 4, PD-L1 deficiency led to lower *C. trachomatis* levels after primary transcervical infection but did not alter *C. trachomatis* levels during secondary infection. PD-1 deficient mice also showed lower *C. trachomatis* levels after primary transcervical infection. I sought to determine if PD-1 deficiency altered secondary bacterial clearance. I transcervically infected mice, allowed the mice to recover for four weeks before transcervically reinfecting these mice. Five days post secondary infection I measured bacterial levels in the uterus using qPCR (**Figure B-1**). Similar to my observation with PD-L1 deficient mice, there was no difference in bacterial levels during secondary infection of WT and PD-1 deficient mice.

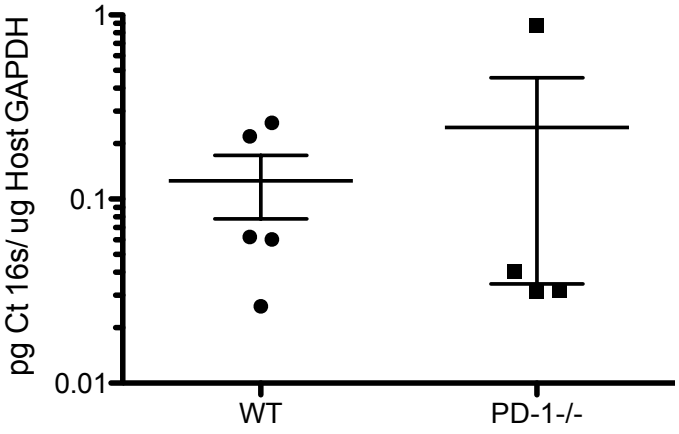

Figure B-1 Bacterial burden during secondary infection of PD-1 deficient mice. WT and PD-1 deficient mice were transcervically infected with 10^6 IFU of *C. trachomatis* and allowed to recover for 4 weeks. Five days after secondary infection, bacterial levels in the uterus were measured by qPCR and normalized to host GAPDH levels. Error bars represent SEM.

I hypothesized that PD-L1 engages PD-1 to shift the $CD8^+$ T cells to a $T_{cm/n}$ phenotype and impair *C. trachomatis* clearance. In order to test this hypothesis I wanted to determine if PD-1 deficiency recapitulated the $CD8^+$ T cell phenotype observed in PD-L1 deficient mice. I therefore examined the $CD8^+$ T cell phenotype in the genital tracts of WT and PD-1 deficient mice during primary infection, memory, and secondary infection. Similar to observations in PD-L1 deficient mice, the $CD8^+$ T cell response in PD-1 deficient mice was shifted toward the T_{em} phenotype at all time points tested (**Figure B-2)**.

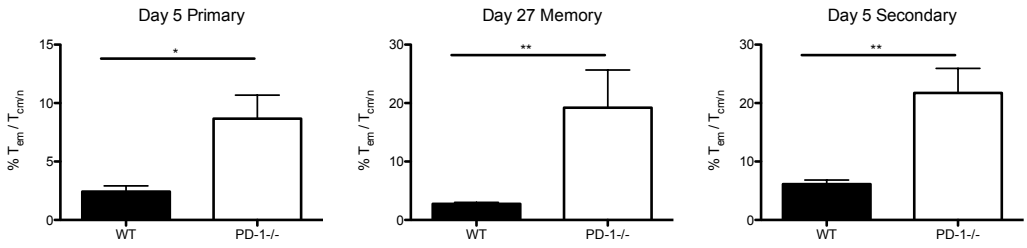

Figure B-2 PD-1 deficiency increases the ratio of T_{em} to $T_{cm/n}$ $CD8^+$ T cells during *C. trachomatis* infection. WT and PD-1 deficient mice were transcervically infected with 10^6 IFU of *C. trachomatis*. At indicated timepoints, uteri were harvested and stained for CD62L and CD127 expression. $CD8^+$ memory populations were gated on live, $CD90.2^+$, $CD127^+$, CD4-, $CD8^+$ cells and either $CD62L^+$ ($T_{cm/n}$) or $CD62L^-$ (T_{em}). The percentage of T_{em} and $T_{cm/n}$ cells of the total $CD8^+$ T cell population in the uterus was measured. Bars represent mean ratios of percentage of $T_{em}/T_{cm/n}$ of 4-5 mice per group and error bars represent SEM. *p<0.05, **p<0.01 using Mann- Whitney Test.

PD-L1 deficiency shifts the $CD8^+$, but not the $CD4^+$, T_{em} to $T_{cm/n}$ ratio

PD-L1 deficiency leads to a significant increase in the ratio of T_{em} to $T_{cm/n}$ cells. However, I wanted to determine if this was due to a total increase in the number of

memory $CD8^+$ T cells present in PD-L1 deficient mice. At the indicated time points I measured the total number of memory $CD8^+$ T cells found in the uteri of WT and PD-L1 deficient mice. The total number of $CD127^+$ memory $CD8^+$ T cell cells is comparable between WT and PD-L1 deficient mice (**Figure B-3**).

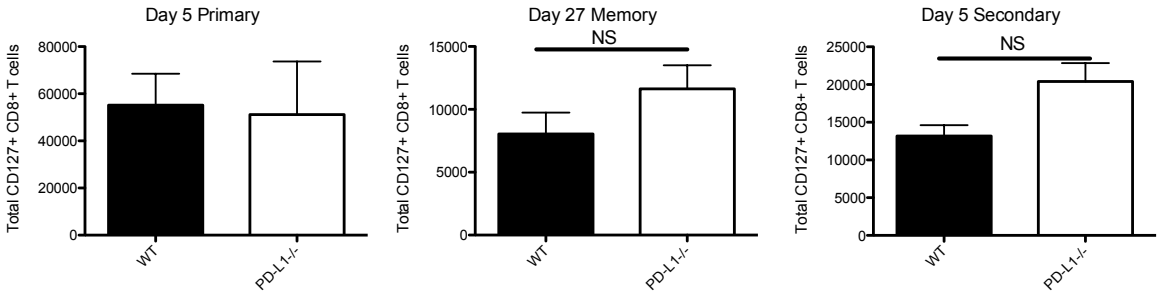

Figure B-3 PD-L1 expression does not alter the total number of memory $CD8^+$ T cells found in the uterus. WT and PD-1 deficient mice were transcervically infected with 10^6 IFU of *C. trachomatis*. At indicated timepoints, uteri were harvested and stained for memory marker CD127. $CD8^+$ memory populations were gated on live, $CD90.2^+$, $CD127^+$, $CD4^-$, $CD8^+$ cells. Bars represent mean of total number of memory $CD8^+$ T cells from 4-5 mice per group and error bars represent SEM.

When I examined the total numbers of T_{em} and $T_{cm/n}$ cells in WT and PD-L1 deficient mice I consistently observe increases in the total numbers of T_{em} cells in PD-L1 deficient mice, although these increases are not statistically significant (**Figure B-4**). Together these data support that PD-L1 deficiency does not alter the number of memory $CD8^+$ T cell present in the uterus, but in a given host shifts the ratio of memory $CD8^+$ T cells by increasing the number of T_{em} cells and decreasing the number of $T_{cm/n}$ cells.

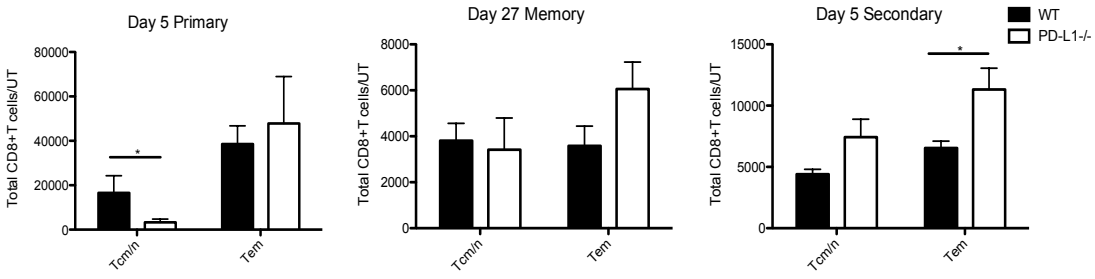

Figure B-4 Total numbers of $T_{cm/n}$ and T_{em} cells in WT and PD-L1 deficient mice. WT and PD-1 deficient mice were transcervically infected with 10^6 IFU of *C. trachomatis*. At indicated timepoints, uteri were harvested and stained for CD62L and CD127 expression. $CD8^+$ memory populations were gated on live, $CD90.2^+$, $CD127^+$, $CD4^-$, $CD8^+$ cells and either $CD62L^+$ ($T_{cm/n}$) or CD62L- (T_{em}). Bars represent mean totals $T_{cm/n}$ and T_{em} of 4-5 mice per group and error bars represent SEM. *$p<0.05$ using Mann-Whitney Test.

It is clear that PD-L1 deficiency shifts the $CD8^+$ T cells towards a $T_{cm/n}$ phenotype. However, I wanted to understand if this shift in phenotype was specific for $CD8^+$ T cells or if it also occurred in the $CD4^+$ T cell population. I therefore examined the ratio of $CD4^+$ T_{em} to $T_{cm/n}$ cells in the genital tracts of infected mice. At all time points tested there was no difference in the ratio of $CD4^+$ T_{em} to $T_{cm/n}$ cells between WT and PD-L1 deficient mice (**Figure B-5).** These data indicate that PD-L1 expression specifically affects the $CD8^+$ T cell population during *C. trachomatis* infection.

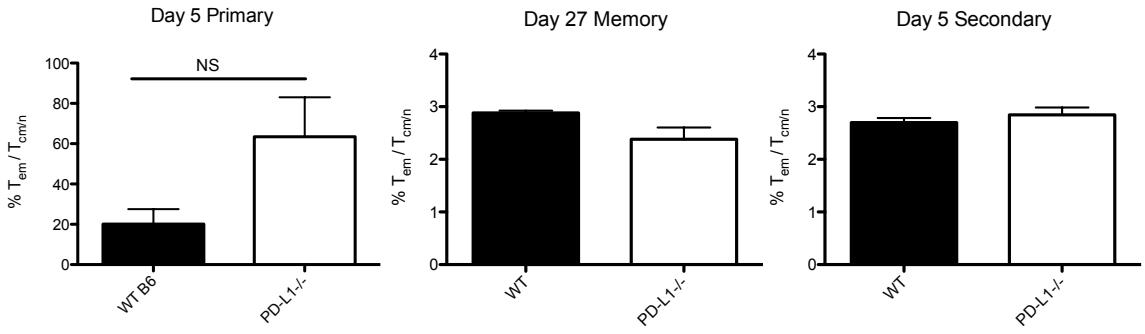

Figure B-5 PD-L1 expression does not shift the ratio of T_{em} to $T_{cm/n}$ $CD4^+$ T cells during *C. trachomatis* infection. WT and PD-L1 deficient mice were transcervically infected with 10^6 IFU of *C. trachomatis*. At indicated timepoints, uteri were harvested and stained for CD62L and CD127 expression. $CD4^+$ memory populations were gated on live, $CD90.2^+$, $CD127^+$, $CD4^+$, $CD8^-$ cells and either $CD62L^+$ ($T_{cm/n}$) or CD62L- (T_{em}). The percentage of T_{em} and $T_{cm/n}$ cells of the total $CD4^+$ T cell population in the uterus was measured. Bars represent mean ratios of percentage of $T_{em}/T_{cm/n}$ of 4-5 mice per group and error bars represent SEM.

PD-1 expression on $CD8^+$ T cells

In Chapter Three I demonstrated that PD-1 expression was upregulated on CD8+ T cells in the dLNs following resolution of *C. trachomatis* infection. PD-1 expression was extremely low and although there was a significant increase in PD-1 expression on antigen-experienced CD8+ T cells, it was still low. The representative histograms demonstrate the increase in PD-1 expression on CD8+ T cells following *C. trachomatis* infection (**Figure B-6**)

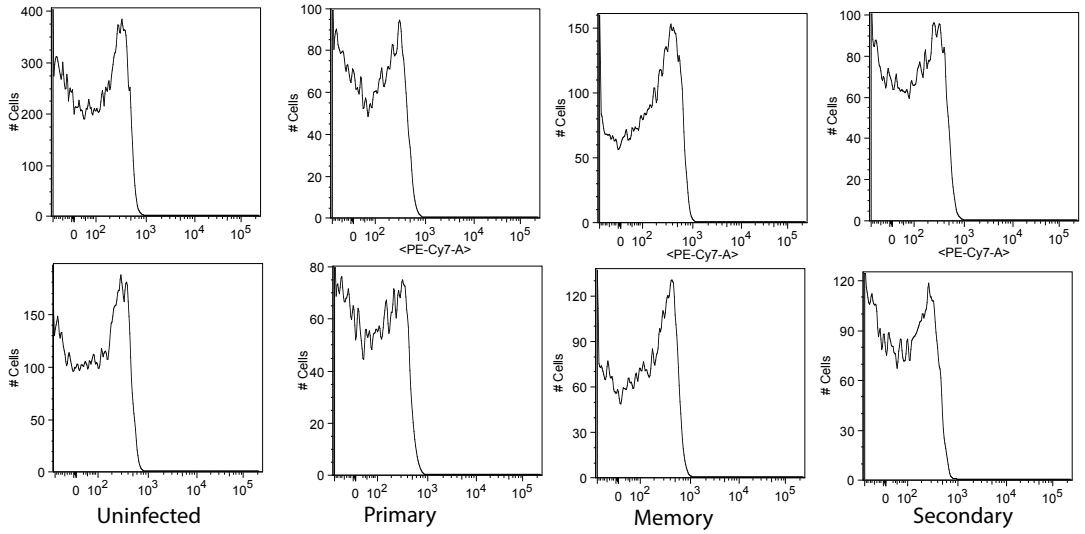

Figure B-6 PD-1 expression is upregulated on CD8$^+$ T cells in the dLNs following *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis*. PD-1 expression was measured by flow cytometry on CD8$^+$ T cells in the dLNs five days post primary infection, 27 days post primary infection (memory), or five days post secondary infection. Shown are 2 representative histograms of PD-1 expression CD3$^+$ CD8$^+$ T cells of the dLNs.

Chapter Four demonstrated that PD-1 expression limits *C. trachomatis* clearance in the genital tract. However, bacterial levels measured in PD-1, on the Thy1.1 background, and Thy1.1 (WT) mice were lower than typically measured in PD-L1, Thy1.2 background, and Thy1.2 (WT) mice. Interestingly, PD-1 expression is significantly lower on CD8$^+$ T cells from Thy1.1 mice and this may partially explain the lower bacterial levels measured during infection (**Figure B-7**).

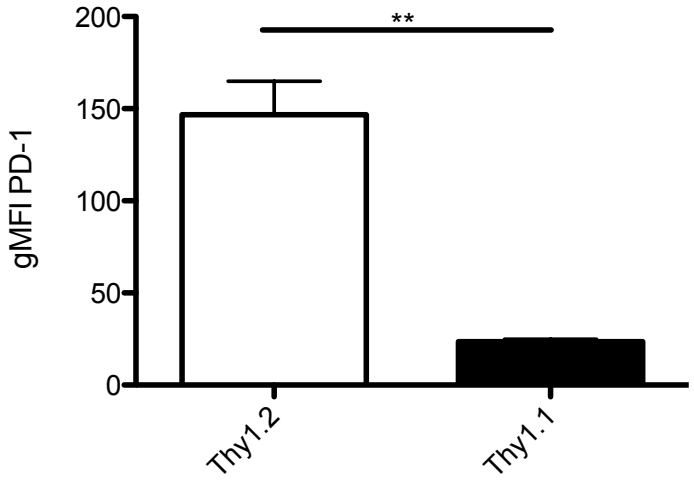

Figure B-7 PD-1 expression is lower on CD8$^+$ T cells in Thy1.1 mice compared to Thy1.2 mice following *C. trachomatis* infection. Mice were transcervically infected with 10^6 IFU of *C. trachomatis.* PD-1 expression was measured by flow cytometry on CD8$^+$ T cells in the spleen five days post primary infection. Bar graphs show gMFI of PD-1 expression on CD3$^+$ CD8$^+$ T cells in the spleen; shown is the average of 5 mice per time point and error bars represent SEM. Statistical significance is indicated by *p<0.05 and **p<0.01 by Mann-Whitney Test.

T cell depletion in WT and PD-L1 deficient mice

In Chapter Four I demonstrated that CD8$^+$ T cell depletion in PD-L1 deficient mice abrogated protection against primary transcervical *C. trachomatis* infection. To confirm depletion, I examined the CD8$^+$ T cell population in the uterus, dLNs and spleen (**Figure B-8**). I also examined the CD4$^+$ T cell population to ensure that antibody depletion did not significantly affect the CD4$^+$ T cell population (**Figure B-8**). Antibody treatment with the anti-CD8 antibody reduced the CD8$^+$ T cell population between 10 and 100 fold in all organs tested in both WT and PD-L1 deficient mice. Additionally, the CD4$^+$ T cell population was not significantly impacted. These data confirm that CD8$^+$ T

cell depletion in PD-L1 deficient mice abrogates protection against *C. trachomatis* infection.

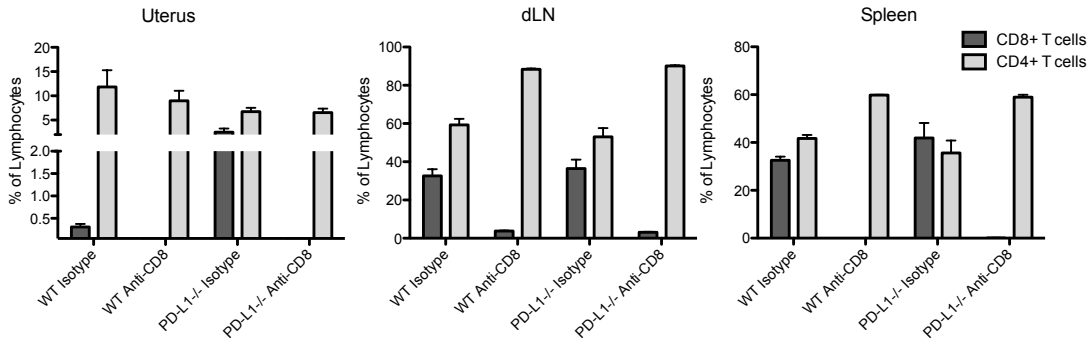

Figure B-8 CD8$^+$ T cells are depleted upon antibody treatment. WT and PD-L1 deficient mice were treated with anti-CD8 depleting antibody or isotype control antibody 3 days prior to and 2 days after primary infection. Mice were infected with 10^6 IFU of *C. trachomatis*, and five days post infection CD8$^+$ and CD4$^+$ T cells were examined in the uterus, dLNs, and spleen.

In Chapter Four I demonstrated that in PD-L1 deficient mice the CD8$^+$ T cell population was able to compensate for the lack of CD4$^+$ T cells and confer protection against secondary transcervical *C. trachomatis* challenge (**Chapter 4 Figure 4-11**). To confirm depletion of CD4$^+$ T cells I measured the CD4$^+$ T cell population in the uterus, dLNs and spleen of WT and PD-L1 deficient mice. The CD4$^+$ T cell population was reduced 10 to 100 fold in all organs tested of mice treated with anti-CD4 antibody (**Figure B-9**). Additionally the CD8$^+$ T cell population was not negatively impacted by antibody treatment. These data confirm that CD4$^+$ T cell depletion was successful and that protection observed in CD4-depleted PD-L1 deficient mice is not due to the presence of CD4$^+$ T cells.

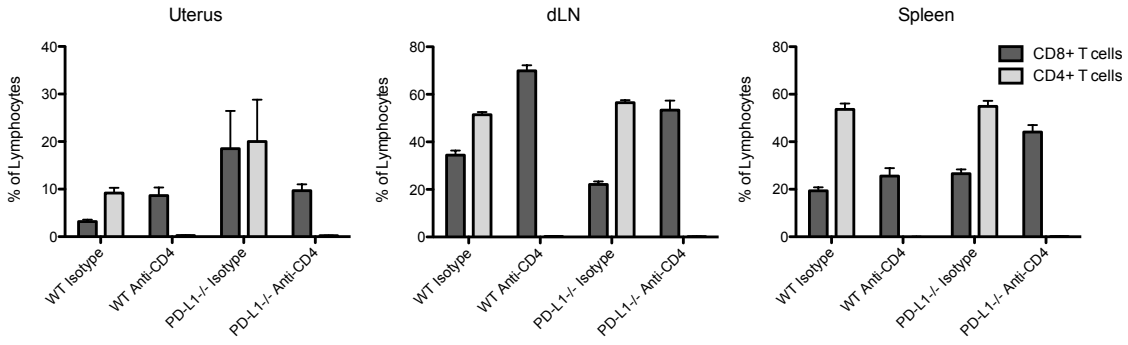

Figure B-9 CD4$^+$ T cells are depleted upon antibody treatment. WT and PD-L1 deficient mice were treated with anti-CD4 depleting antibody or isotype control antibody 3 days prior to and 2 days after secondary infection. Mice were infected with 10^6 IFU of *C. trachomatis*, and five days post infection CD8$^+$ and CD4$^+$ T cells were measured in the uterus, dLNs, and spleen.

Taken together the data from this chapter support the conclusions presented in Chapter Four. Importantly the data indicating that PD-1 deficiency recapitulates the phenotype of PD-L1 deficiency provides additional evidence that the PD-1/PD-L1 interaction specifically shifts the memory CD8$^+$ T cell population to the T_{em} phenotype. In addition, the successful CD8$^+$ and CD4$^+$ T cell depletions supports the data indicating that CD8$^+$ T cells are an important protective population in PD-L1 deficient mice during transcervical *C. trachomatis* infection.